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THE JOURNAL
OF
BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

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VOLUME 154
BALTIMORE
1944

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THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, Inc.
Baltimore 2, U. S. A.

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BIOLOGICAL CONVERSION OF ACTIVE NON-QUINONES OF VITAMIN K TO THE QUINONE FORM

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(Received for publication, January 25, 1944)

As soon as it was announced by McKee *et al.* (1) that vitamins K_1 and K_2 are quinones, Doisy and his coworkers (2) tested various compounds for vitamin K activity and stated (3) that, "Only 1,4-naphthoquinones and compounds which upon oxidation in the organism might yield 1,4-naphthoquinones showed activity." Such non-quinones as methyltetralones (4, 5) and 4-amino-2-methyl-1-naphthol (2, 6–8) as well as such esters and ethers of 2-methyl-1,4-naphthohydroquinone as the diphosphate (6, 9–11) and 2-methyl-1,4-dimethoxynaphthalene (12) have now been shown to possess high vitamin K potencies. On the other hand Fieser *et al.* (13) found that compounds, which due to substituents in the 1 or 4 position could not give 1,4-quinones, are inactive.

An investigation of the question of associating the activity of these compounds with their conversion has been extended by metabolism studies in which the compounds were administered to fowls and rabbits, and their conversion to quinone estimated by determining colorimetrically the amount of methylnaphthoquinone in the urine.

Colorimetric Analysis of 2-Methyl-1,4-naphthoquinone

2-Methyl-1,4-naphthoquinone was found to respond to the Craven (14) ethyl cyanoacetate test for quinones by giving a deep blue color which faded rapidly. It occurred to us that perhaps the color could be stabilized by reducing the alkalinity, thereby making it applicable to colorimetric measurements. It was found that at a pH of 10.5 the color is stable for at least 25 minutes. For example, the color was developed with 32 γ of 2-methyl-1,4-naphthoquinone according to the procedure described below. 5 minutes later the transmission reading was made with a Cenco-Sheard spectrophotelometer and was found to be 69 per cent. 25 minutes later the transmission was still 69 per cent. The intense blue color exhibits maximum light absorption in the region of 570 mμ (Fig. 1), and the intensity is proportional to the concentration of 2-methyl-1,4-naphthoquinone in compliance with Beer's law (Fig. 2). 8 to 10 γ may be detected and measured satisfactorily (Figs. 2 and 3).

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Application to Alcoholic Solutions—The test for 2-methyl-1,4-naphthoquinone in ethyl alcohol is made by treating 1 cc. of the solution with 3 drops of ethyl cyanoacetate and 5 cc. of a sodium borate buffer of pH 10.5 (15) in a photometer tube. Several minutes are allowed for the color to develop fully and the intensity is measured in a photelometer with an orange filter transmitting maximally at 590 mμ. Calculation is made by reference to a calibration curve prepared with various known concentrations of 2-methyl-1,4-naphthoquinone (Fig. 3).

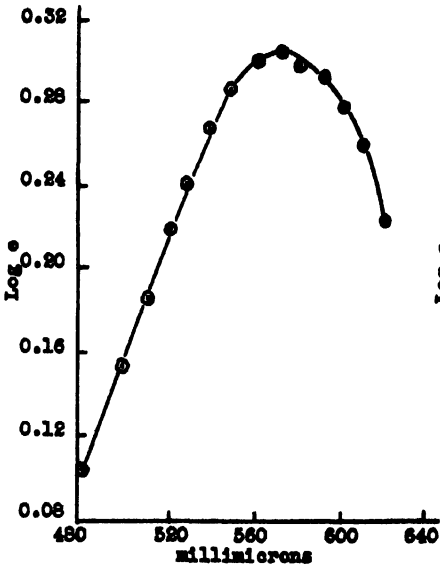

FIG. 1

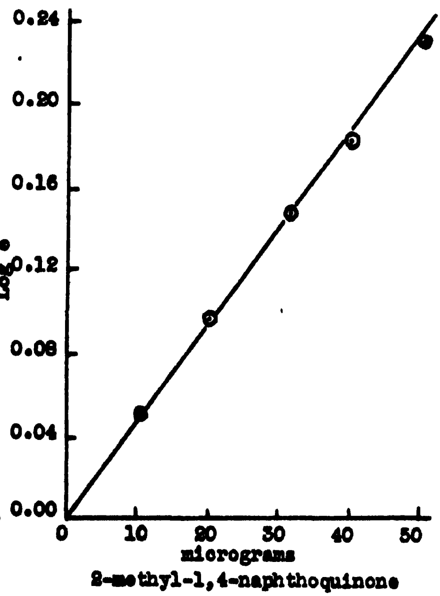

FIG. 2

FIG. 1. Absorption curve of the ethyl cyanoacetate-2-methyl-1,4-naphthoquinone color reaction. Measurements were made with a Cenco-Sheard spectrophotelometer.

FIG. 2. Extinction-concentration curve of the ethyl cyanoacetate-2-methyl-1,4-naphthoquinone color reaction. The color was developed with 1 cc. of alcoholic solution of the quinone, 3 drops of ethyl cyanoacetate, and 5 cc. of 0.2 M sodium borate buffer, pH 10.5. Measurements were made with a Cenco-Sheard spectrophotelometer.

Application to Urine Analysis—We found that 2-methyl-1,4-naphthoquinone administered to animals is excreted in the urine in a conjugated form which must be hydrolyzed before it responds to the color test. The following procedure was used in the metabolism studies.

A measured volume of urine was placed in a round bottom flask equipped with a ground glass joint. Concentrated hydrochloric acid (10 cc. of HCl to 100 cc. of urine) was added, the flask fitted with a condenser, and the urine boiled for 30 minutes and allowed to cool to room temperature. Since

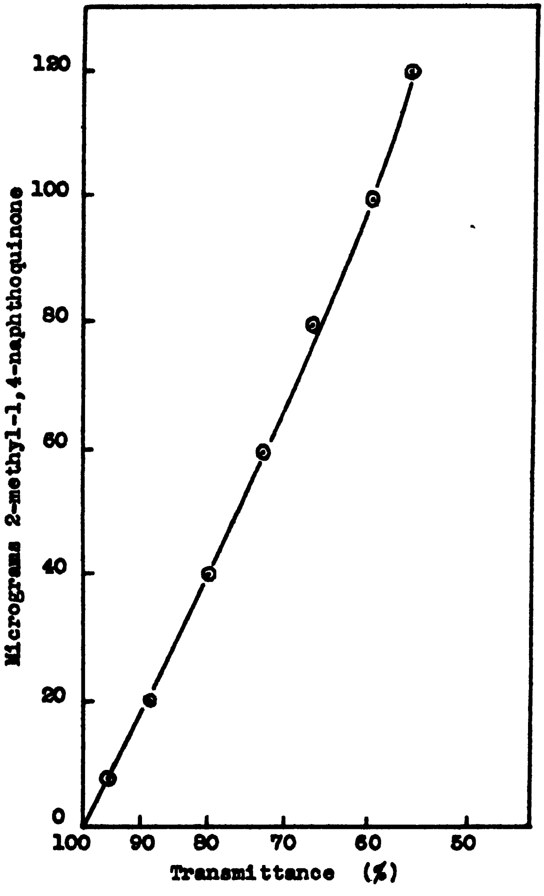

FIG. 3. Calibration curve for 2-methyl-1,4-naphthoquinone, obtained with a photelometer for routine use equipped with a No. 348 filter, transmitting maximally at 590 mμ.

2-methyl-1,4-naphthoquinone sublimes rather easily, precautions were taken to rinse the bore of the condenser with small portions of ether before it was disconnected. The cooled urine was nearly saturated with sodium chloride to prevent heavy emulsions from forming when shaken with ether. The urine was extracted three times with 100 cc. portions of ether in the separatory funnel. The extracts were combined and gently agitated for several hours by occasionally shaking or with a gentle stream of air passing through the solution. This was done to insure oxidation of any methylnaphthohydroquinone which was assumed to be formed by the acid hydrolysis.

The ether solution was next extracted with an equal volume of 0.05 N NaOH solution, which removed most of the pigments. This was followed by one washing with distilled water. The ether phase was drained into a distilling flask, and the ether evaporated. The residue was dissolved in absolute alcohol and transferred by means of a pipette to a volumetric flask of appropriate volume in which it could be properly diluted with additional alcohol.

The color was then developed in 1 cc. portions of the alcohol solution. These solutions usually still contained some ether-soluble pigments. Several cc. of ether were added to the tube containing the colored solution and the contents thoroughly shaken. All of the color due to the quinone remained in the aqueous phase, while the interfering pigments were extracted by the ether. The tubes were centrifuged if the solutions were turbid, and photelometric readings were made. The recovery of known amounts of 2-methyl-1,4-naphthoquinone added to urine ranged from 80 to 90 per cent. Of 0.2, 0.6, 1.0, 1.3, 8.0, and 16.0 mg. of the quinone added to 100 cc. samples of urine, 90, 91, 90, 84, 80, and 80 per cent respectively were recovered. Decreasing the concentration of the acid or of the time for hydrolysis, or performing the hydrolysis in the absence of light, did not increase the efficiency of the recoveries.

Metabolism—It had been observed that when 2-methyl-1,4-naphthoquinone was administered to rabbits 31 to 42 per cent of it could be recovered from the urine collected in metabolism cages over periods of 18 hours after treatment (Table I). The quinone was suspended in benne oil for intramuscular and subcutaneous injections, while oral administrations were made by emulsifying the quinone with oil or milk and feeding by stomach tube.

It seemed probable that, if non-quinone antihemorrhagic compounds are converted to methylnaphthoquinone, they would be excreted in the same conjugated form as administered 2-methyl-1,4-naphthoquinone. Conversion studies of 4-amino-2-methyl-1-naphthol, 2-methyl-1-tetralone, 2-methyl-1,4-dimethoxynaphthalene, and tetralithium 2-methyl-1,4-naphthohydroquinone diphosphate in the rabbit and of 2-methyl-1-tetralone and 2-methyl-1,4-dimethoxynaphthalene in the chicken[1] are here reported.

---

[1] Year-old cocks were provided with an artificial anus by the surgical procedures described by Volts (17). In this way large volumes of urine free from excreta were collected. The author wishes to express his gratitude to Dr. N. J. Wade who performed the operations.

The water-soluble compounds were given intravenously and the others were administered in oil or 30 per cent alcohol by the routes indicated in Table II.

The animals were kept in metabolism cages and the urine was collected in flasks containing chloroform (and 3 cc. of concentrated HCl in the diphosphate experiments to prevent hydrolytic action of phosphatases on any diphosphate which might possibly have been excreted unchanged). It was found that the acid in this concentration did not hydrolyze the diphosphoric acid ester. In fact, the diphosphate proved to be stable even to boiling for 30 minutes in 100 cc. of urine or water to which had been added 10 cc. of concentrated HCl.

TABLE I

Recovery of 2-Methyl-1,4-naphthoquinone from Hydrolyzed Urine Following Administration of Quinone to Rabbits

Rabbit No.	Administration	Dosage	Methylnaphthoquinone recovered	
		mg.	*mg.*	*per cent*
1	Intravenous*	5	1.6	31
2	" *	6	2.0	33
3	Intramuscular (oil)	20	7.5	42
4	Subcutaneous "	25	6.7	32
5	Oral (milk emulsion)	40	15.7	39.3
6	" (oil ")	500	189.0	37.8
7	" " "	500	180.0	36.0

* Aqueous solution of a bisulfite addition product (16) of 2-methyl-1,4-naphthoquinone, Abbott.

An ether extract of hydrolyzed normal rabbit urine contains a substance which develops a slight bluish color with ethyl cyanoacetate, but which is without antihemorrhagic activity. The residue of hydrolyzed normal rabbit urine was dissolved in benne oil and administered orally to vitamin K-deficient chicks. Each of six chicks received a dose equivalent to the color produced by 6 γ of 2-methyl-1,4-naphthoquinone. The clotting time (19) was not reduced to normal in any of the cases. The urine of chickens contains less interfering substance.

Adequate control values were established on urine samples collected before the animals were treated. An amount of material equal to that administered was added to these samples, and the urines hydrolyzed and assayed. Thus any assay value above the control represented quinone which had been formed through biological conversion of the administered compound, and not that formed by chemical conversion of any of the substance possibly excreted unchanged.

Results

Administered 4-amino-2-methyl-1-naphthol produced large quantities of methylnaphthoquinone in the urine. The aminonaphthol is readily oxidized to the quinone when allowed to stand in solution exposed to air or when heated with 1 N HCl for half an hour. However, there are several reasons for believing that the methylnaphthoquinone found in the hydrolyzed urine following the administration of the aminonaphthol was not the

TABLE II

Conversion of Vitamin K Active Non-Quinones to Methylnaphthoquinone

Compound	Animal	Administration	Dose*	2-Methyl-1,4-naphthoquinone recovered	Control	Minimum amount converted†	
			mg.	*mg.*	*mg.*	*mg.*	*per cent*
4-Amino-2-methyl-1-naphthol‡	Rabbit	Intravenous	6.0	2.50		2.50	41.70
	"	"	14.0	3.52		3.52	25.14
2-Methyl-1-tetralone	Chicken	Oral (oil)	43.0	3.00		3.00	7.00
	"	" "	75.0	5.80		5.00	7.75
	Rabbit	Subcutaneous (oil)	21.5	1.12	0.12	1.00	4.65
	"	Intramuscular "	21.5	1.17	0.19	0.98	4.55
	"	Oral (oil)	54.0	2.52	0.44	2.08	3.70
	"	" "	54.0	1.81	0.59	1.22	2.26
2-Methyl-1,4-dimethoxynaphthalene	Chicken	" "	42.0	0.41	0.09	0.32	0.76
	"	" "	42.0	0.51	0.21	0.30	0.73
	"	" (30% alcohol)	51.0	0.62	0.21	0.41	0.80
	"	" (30% ")	84.0	1.65	0.33	1.32	1.57
	"	Intramuscular (oil)	84.0	0.75	0.33	0.42	0.50
	Rabbit	Oral (30% alcohol)	10.0	0.45	0.70		
	"	" (30% ")	15.0	0.42	0.55		
2-Methyl-1,4-naphthohydroquinone diphosphate	"	Intravenous	6.0	2.0		2.0	33.3
	"	"	13.4	5.1	0.5	4.6	34.3
	"	"	13.4	4.1	0.5	3.6	27.0
	"	"	17.9	5.4	0.8	4.6	25.7
	"	"	44.7	11.8		11.8	26.5
	"	"	44.7	11.9	1.3	10.6	23.7

* Equivalent to 2-methyl-1,4-naphthoquinone.

† These values are considered to represent the minimum amount converted for the following reasons. (*a*) The recovery of known amounts of 2-methyl-1,4-naphthoquinone added to urine was only 80 to 90 per cent efficient. (*b*) According to Scudi and Buhs (18) 2-methyl-1,4-naphthoquinone undergoes addition reactions with and is adsorbed by the plasma proteins and is in part converted to phthiocol. (*c*) It is not known whether more than one conjugated product is excreted and, if so, whether 2-methyl-1,4-naphthoquinone is completely liberated under the conditions of hydrolysis that were used.

‡ As the crystalline hydrochloride containing 0.5 M ethanol.

result of the hydrolytic acid treatment on the amine excreted in an unchanged form, but that it had undergone a change in the organism prior to its elimination. (*a*) The urine was extracted with ether before it was subjected to hydrolysis. This should have removed any unchanged 4-amino-2-methyl-1-naphthol. (*b*) The substance excreted in the urine was more stable to free quinone formation when allowed to stand in the urine at room temperature than was the amine when added to normal urine. A dose of 4-amino-2-methyl-1-naphthol, equivalent to 14 mg. of 2-methyl-1,4-naphthoquinone, was administered to a rabbit by intravenous injection. About 8 hours later the rabbit urinated. At this time an equal amount of the amine was added to a control sample of urine collected from the same rabbit just before the injection, and the two samples were allowed to stand at room temperature for 20 hours. Then each was extracted with ether. Nearly all of the 4-amino-2-methyl-1-naphthol which was added to the urine had been oxidized, as approximately 13 mg. of 2-methyl-1,4-naphthoquinone were found in the ether extract. However, only a small amount of quinone (0.4 mg.) was extracted from the urine of the treated rabbit. Following hydrolysis of the urines, 3.12 mg. of methylnaphthoquinone were recovered from the test sample, while only 0.27 mg. was found in the urine to which the amine had been added directly.

As much as 7.75 per cent of the administered tetralone was detected in chicken urine as methylnaphthoquinone. Conversion took place in both species.

The amount of methylnaphthoquinone in the hydrolyzed rabbit urine following the administration of the diphosphoric acid ester approached the quantities recovered in the experiments with methylnaphthoquinone. This compound is apparently hydrolyzed in the body and excreted in the same labile conjugated form as 2-methyl-1,4-naphthoquinone.

2-Methyl-1,4-dimethoxynaphthalene was converted by the chicken to a very small extent. 0.50 to 1.57 per cent of the administered material was recovered as methylnaphthoquinone.

SUMMARY

Craven's ethyl cyanoacetate color test for quinones has been applied to the development of a method for the determination of 2-methyl-1,4-naphthoquinone in the urine. It has been found that such potent antihemorrhagic compounds as 4-amino-2-methyl-1-naphthol, 2-methyl-1,4-naphthohydroquinone diphosphate, and 2-methyl-1-tetralone are extensively converted to methylnaphthoquinone in the organism and excreted in a combined form.

The author wishes to express his gratitude to Dr. Edward A. Doisy for his interest and helpful suggestions toward carrying out this research.

Moreover, he wishes to acknowledge financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

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THE EFFECT OF THE NATURE AND LEVEL OF PROTEIN AND AMINO ACID INTAKE UPON THE ACCUMULATION OF FAT IN THE LIVER

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(Received for publication, February 24, 1944)

Several groups of workers have attempted to account for the lipotropic action of casein and certain other proteins. In 1937 Tucker and Eckstein (1) discovered the lipotropic effect of methionine and suggested that the lipotropic action of a ration containing 30 per cent casein is "a resultant effect of the simultaneous opposing influences of the cystine and methionine of the diet." Further studies by Tucker and Eckstein (2) led them to consider the possibility that other amino acids may exert the same effects as methionine and cystine. Subsequently they published data in support of their original hypothesis that the lipotropic action of casein could be completely accounted for by its methionine and cystine contents (3, 4). On the other hand, data published by Channon *et al.* (5, 6) and by Best and Ridout (7) have indicated that other factors are involved. As an outgrowth of these studies conflicting conclusions have been reached concerning the relative lipotropic efficiency of methionine when fed as the free amino acid and as a constituent of an intact dietary protein (3–5, 7).

There are several complicating factors, such as the total caloric intake, the nitrogen balance, and the dietary level of the essential amino acids, which have not received adequate consideration in some of the experiments described to date. The apparent discrepancies mentioned above may have some simple explanation, since no group has duplicated exactly the dietary conditions used by another, particularly in regard to the *level* and *nature* of the protein in the basal diet. While considerable work has already been done on the effect of these two factors, there are still many gaps in our knowledge of this phase of the subject. The following experiments were designed to examine the influence of the nature and level of the dietary protein and of certain essential amino acids upon the lipotropic efficiency of methionine.

EXPERIMENTAL

For the experiments to be described a diet was desired which would be as low as possible in lipotropic factors, but which would still be capable of maintaining the body weight of the rats. The suitability of gelatin as the basal protein was considered, since data in the literature indicate that it has no lipotropic influence (8). Moreover, the better grades are *very* low in methionine and cystine, if not completely free of them. It was realized that this deficient protein would require supplementation either by essential amino acids or by a complete protein. A preliminary experiment was therefore conducted to determine the minimal ratio of casein to gelatin (at a total protein level of 20 per cent) which would just maintain the body weight of young adult rats on a high fat diet. Twelve diets containing 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, and 20 per cent casein, respectively, were prepared. All diets contained 40 per cent beef dripping, 33 per cent cane sugar, 5 per cent salt mixture (McCollum, No. 185 (9)), and agar 2 per cent. A cod liver oil concentrate (500,000 I.U. of vitamin A and 50,000 I.U. of vitamin D per gm.) was added at a 0.015 per cent level. All rats received daily subcutaneous injections of 0.5 cc. of a solution in physiological saline of 25 γ of thiamine chloride, 20 γ of riboflavin, 20 γ of pyridoxine, 100 γ of calcium pantothenate, and 100 γ of nicotinic acid. Ten rats (150 to 200 gm.) were fed on each diet for 21 days. Weight changes were recorded twice per week. The animals were killed by a blow on the head. The livers were analyzed for total fatty acids by the usual saponification procedure, followed by acidification and extraction with petroleum ether. The findings presented in Table I (Experiment A) show that 8 per cent casein with 12 per cent gelatin sufficed to maintain body weight and that this quantity of casein did not reduce the liver fat. The findings were confirmed by a repeat experiment (performed 3 months later, in August, 1942) on groups of ten rats under identical conditions at casein levels of 0, 1, 2, 3, 4, 5, 6, 8, and 10 per cent. The data presented in Table I (Experiment B) show that an 8 per cent casein + 12 per cent gelatin mixture practically maintains body weight. If one averages the data obtained from all twenty animals used at each casein level in Experiments A and B, the weight loss at 6 per cent casein is 6.3 per cent; at 8 per cent no loss or gain is noted and at 10 per cent the gain is 2.1 per cent.

The gelatin actually used was reported to be practically free from organic sulfur. The colorimetric test for methionine proposed by Sullivan and McCarthy (10) appeared to be negative as also was the cyanide-nitroprusside test for cystine, and so the gelatin was fed without further examination.[1] Later it was analyzed for total and inorganic sulfur and 1.07 and 0.86 per cent, respectively, were found. This led to the determination of the sulfur distribution by the Kassell and Brand (12) modification of Baernstein's (13) method. Methionine found by volatile iodide was 1.02 per cent, and by thiolactone 0.84 per cent. Only 0.03 per cent cystine was found. Inorganic sulfur by this method was 0.88 per cent. Unfortunately, the presence of this not negligible amount of methionine was discovered only after the conclusion of the feeding experiments. However, even 20 per cent of this gelatin in the diet did not produce any detectable lipotropic effect.

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[1] Subsequently, after methionine was found to be present by the HI method, application of the colorimetric test as later described by McCarthy and Sullivan (11) indicated the presence of methionine. Apparently the omission of thorough shaking after the final step, not mentioned earlier (10), is essential for the full development of the color. A quantitative determination by this method gave a figure of 0.5 per cent methionine in gelatin.

TABLE I

Effect on Body Weight and Liver Fat of Increasing Casein-Gelatin Ratio in High Fat Diet

Ten rats (initial weight 150 to 200 gm.) in each group; duration of test, 21 days.

Experiment and diet No.	Casein: gelatin	Change in weight	Food ingested	Liver fat		
				As per cent liver weight		As per cent body weight, average
				Range	Average	
	per cent	*per cent*	*gm per rat per day*			
A-1	0:20	−19.7	9.1	35.2–25.1	28.3	1.61
A-2	1:19	−20.1	11.0	32.2–13.2	23.7	1.44
A-3	2:18	−16.3	10.4	35.3–22.8	29.4	1.88
A-4	3:17	−13.2	10.1	35.2–20.1	27.9	1.72
A-5	4:16	−7.9	12.8	37.5–23.4	30.2	1.87
A-6	5:15	−7.9	11.7	37.1–19.8	29.0	1.69
A-7	6:14	−4.7	10.7	32.9–14.3	26.5	1.35
A-8	8:12	+0.9	10.1	35.8–12.3	26.0	1.49
A-9	10:10	+2.1	10.1	32.8–13.0	21.9	1.12
A-10	12:8	+8.6	9.1	32.2–10.2	18.5	0.82
A-11	16:4	+4.5	8.3	24.8– 7.5	11.3	0.46
A-12	20:0	+6.7	7.5	10.3– 7.0	8.6	0.32
B-1	0:20	−25.0	8.4	29.4–15.8	23.9	1.14
B-2	1:19	−19.2	9.1	30.6–20.8	25.9	1.42
B-3	2:18	−18.2	9.9	35.0–12.0	24.1	1.33
B-4	3:17	−15.8	10.1	33.6–15.3	24.9	1.43
B-5	4:16	−16.4	10.9	35.7–17.1	25.1	1.38
B-6	5:15	−12.6	11.0	34.5–21.0	26.9	1.53
B-7	6:14	−7.9	11.4	33.3–18.5	27.4	1.63
B-8	8:12	−0.9	12.0	35.7–22.2	26.6	1.56
B-9	10:10	+2.1	11.3	34.2–12.7	28.1	1.73

Diet 2 (8 per cent casein + 12 per cent gelatin) in the following experiment thus contained 254 + 122 = 376 mg. of methionine per 100 gm. and Diet 3 (19.7 per cent gelatin + 254 mg. of methionine) contained 201 + 254 = 455 mg. of methionine. The methionine inadvertently included in Diet 3 therefore resulted in an excess of 21 per cent over that present in

Diet 2. Although the experiments involving the assay of the lipotropic effect of casein on the basis of its methionine and cystine contents are disturbed, the effects of supplemental essential amino acids upon liver fat are quite clear cut despite the imbalance of the methionine contents of some of the diets.

The experiment was originally designed to investigate the fundamental question as to whether methionine and cystine alone account for the lipotropic action of casein, and to determine whether a deficiency in essential amino acids affects the results. It seemed desirable to determine whether the effects were similar on a diet capable of maintaining body weight and on one permitting an appreciable weight loss. The basal diet used for the experiments was as follows: protein (gelatin), 20 per cent; beef dripping, 40; sucrose, 33; salts (McCollum, No. 185), 5; agar, 2; vitamin A and D concentrate, 0.015 per cent. The B vitamins were given as described in the previous experiment. In the following test diets it will be noted that casein or amino acid supplements were added at the expense of the gelatin.

Since we had determined that a high fat diet containing 12 per cent gelatin + 8 per cent casein will maintain body weight without lowering liver fat, it was decided to use this protein combination for the test diet of one group of rats. Another group of rats was given a ration containing 16 per cent gelatin + 4 per cent casein, which permits a definite weight loss.

Gelatin is not a complete protein, lacking, or being deficient in, the following essential amino acids besides methionine: tryptophane, valine, isoleucine,[2] and threonine.[3] Thus the diet of the group of rats receiving the gelatin supplemented with the quantity of methionine believed equivalent to that in 8 per cent casein differs from the diet of the animals on 12 per cent gelatin + 8 per cent casein in that it lacks the essential amino acids mentioned above. To control any possible effect of these dietary essentials upon the capacity of methionine to lower liver fat, another group of rats was therefore fed 18.7 per cent gelatin plus appropriate quantities of these essential amino acids. Because of the presence of a small amount of cystine in casein, an equivalent quantity of this antilipotropic substance was also added to the diets containing free methionine. The nature of the protein moiety of the experimental diets is shown in Table II.

The diets were fed to two series of rats: Series I consisted of young animals from 110 to 160 gm. in weight; Series II were older animals weighing 230 to 270 gm. Each diet was fed to a group of five rats, the younger ones being on the diet for 28 days, the older ones for only 21 days. The results obtained are shown in Table II.

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[2] Block and Bolling (14) report 1.1 ± 0.2 per cent isoleucine in a commercial grade of gelatin.

[3] Rose (15) found by feeding experiments that gelatin was an exceedingly poor source of threonine. More recently Shinn and Nicolet (16) determined its threonine content to be only 1.5 per cent.

It may be noted that in Series I the rats whose food contained casein (Diet 2) consumed a much larger amount than did the rats of the other groups. Because of this fact one would be led by the work of Griffith and Mulford (17) to expect a greater accumulation of liver fat than if the food intake had been restricted, and this was observed. It is intended, therefore, to repeat this experiment on a paired feeding basis to eliminate as far as possible any effect of difference in food consumption. It may be noted that in the case of thé rats on Diet 4 the food consumption was not appreciably greater than that of the rats on Diet 3; yet the inclusion of the essential amino acids more than doubled the liver fat. In the case of older rats

TABLE II

Effect of Essential Amino Acids upon Apparent Lipotropic Activity of Methionine

Diet 1, basal, 20 per cent gelatin;* Diet 2, 8 per cent casein (British Drug Houses, fat- and vitamin-free)† + 12 per cent gelatin; Diet 3, 0.25 per cent methionine + 0.034 per cent cystine + 19.7 per cent gelatin (*i.e.* the amounts of these amino acids in an 8 per cent casein diet); Diet 4, 0.25 per cent methionine + 0.034 per cent cystine + 0.8 per cent of a mixture‡ of valine, threonine, and isoleucine + 0.16 per cent tryptophane§ + 18.7 per cent gelatin; Diet 5, 4 per cent casein + 16 per cent gelatin; Diet 6, 0.13 per cent methionine + 0.017 per cent cystine + 19.8 per cent gelatin (*i.e.* the amounts of these amino acids in a 4 per cent casein diet).

Diet No.	Series I (rat weight, 110-160 gm)				Series II (rat weight, 230-270 gm.)			
			Liver fat				Liver fat	
	Weight change	Food consumed	Per cent liver weight	Per cent body weight	Weight change	Food consumed	Per cent liver weight	Per cent body weight
	per cent	*gm. per rat per day*			*per cent*	*gm. per rat per day*		
1	−34.0	5.4	25.7	1.54	−17.8	12.3	27.3	1.28
2	+3.3	8.9	34.1	2.60	+4.7	13.7	20.3	0.80
3	−26.4	5.2	14.4	0.76	−13.1	12.3	16.4	0.68
4	−8.3	6.0	32.2	2.23	+2.4	12.3	23.1	1.14
5					−8.4	12.2	28.9	1.59
6					−15.5	11.3	16.9	0.68

* This gelatin contained 1.02 per cent methionine and 0.03 per cent cystine.

† This sample contained 3.17 per cent methionine and 0.42 per cent cystine.

‡ This mixture was obtained from a casein hydrolysate by butyl alcohol extraction and formation of the copper salts of the monoamino acids. The water- and methanol-soluble fraction was freed from copper and extracted with ethanol to remove proline. The resulting fraction, which was used for this work, gave negative tests for methionine and cystine.

§ The tryptophane was isolated from a tryptic digest of casein and gave negative tests for methionine and cystine.

(Series II) the food intake on the different diets varied only slightly and consequently disturbing effects due to this factor are less than in Series I. It is interesting, therefore, that the results in Series II parallel those in Series I.

A comparison of the data for Diets 2 and 3 (Series I) reveals a markedly greater lipotropic effect of methionine when fed as free amino acid than when administered in casein. It is doubtful whether the 21 per cent excess methionine in Diet 3 will account for the large difference. The effect is similar but less marked in older rats (Series II). Comparison of liver fats resulting from Diets 5 and 6 again shows that under these conditions free methionine is apparently more lipotropic than is the bound form. Further, the data indicate that under these conditions the results are similar whether the casein-fed paired group maintains body weight (compare Diets 2 and 3) or loses weight (compare Diets 5 and 6). There is, however, a difference between Diets 2 and 3, other than the condition of the methionine, and in Diet 4 an attempt was made to control this other variable.

The results obtained on Diet 4 are of particular interest. The addition of very small quantities of four essential amino acids lacking or deficient in Diet 3 (but present in Diet 2) has obliterated the superior lipotropic effect of free methionine over that of the amino acid as it occurs in casein. It is possible that the increased efficiency of metabolism in the presence of these essential metabolites leads to a greater demand for labile methyl groups, thus limiting the supply available for lipotropic purposes. Such an explanation is in accord with views repeatedly expressed by Griffith *et al.* (17–19). Whatever the explanation, the data show clearly that when methionine, cystine, and essential amino acids present in casein but absent from or deficient in gelatin are given as a supplement to replace the casein supplement added to the basal gelatin diet the level of liver fat in rats on such a diet approximates that of rats fed the methionine in the form of casein.

The above results demonstrate unequivocally that the lipotropic action of a protein cannot be attributed solely to the amount or ratio of methionine and cystine contained therein. The nutritional value of the protein undoubtedly has a significant effect on its lipotropic action. Furthermore, the possibility cannot be dismissed that there may also be present one or more other amino acids which exert positive or negative lipotropic influences either directly or indirectly. Data recently reported by Channon, Mills, and Platt (6) support this possibility.

It is apparent that in order to account for the lipotropic action of any protein or to get a real comparison of the relative lipotropic efficacy of free and bound methionine, or of any other amino acid, one would have to know accurately the amino acid content of the protein under study and prepare a control diet containing all of these in the same quantities in which they occur in the protein. Few, if any, proteins have been analyzed with sufficient completeness and accuracy to make such a rigid test possible. On the other hand, attempts to approximate these conditions should give more accurate information than has been obtained by previous methods.

It was felt that the experiment should be repeated with synthetic essential amino acids in place of the crude fractions isolated from casein. This procedure would indicate whether the effect obtained was actually derived from the essential amino acids supplied in the fraction isolated from casein or whether some unknown constituent of the crude fraction exerted an influence.

Four groups of ten rats each were given diets exactly as described for Series I and II with the exception that Diet 4 contained the following pure amino acids[4] in place of the isolated mixtures: 1.26 per cent *dl*-valine, 0.26 per cent *d*-isoleucine, 0.70 per cent *dl*-threonine, and 0.144 per cent *l*-tryptophane. These quantities are approximately those believed to be contained in an 8 per cent casein diet. The animals were kept on the diet for 25 days. The findings (Table III), which are wholly in accord with those found in Series I and II as far as effects on liver fat are concerned, confirm the importance of the essential amino acids in a comparison of the lipotropic effects of different diets.

TABLE III

Effect of Essential Amino Acids upon Apparent Lipotropic Activity of Methionine

Series III (rat weight 120 to 180 gm.). The diets in this experiment are the same as described in Table II except that in Diet 4 synthetic amino acids replaced the natural mixture from casein fed in the earlier experiment.

Diet No.	Weight change	Food consumed	Liver fat	
			Per cent liver weight	Per cent body weight
	per cent	*gm. per rat per day*		
1	−29.8	5.8	24.7	1.35
2	−5.9	8.9	30.8	2.50
3	−30.1	5.5	15.0	0.73
4	−16.7	6.4	33.2	2.35

DISCUSSION

The results show that the nature and level of protein intake affect markedly the liver fat of rats on high fat diets. They reveal further that an important factor in this effect is probably the adequacy or otherwise of the essential amino acids in the diet. These findings may help to resolve the apparent discrepancies between the conclusions reached by Best and Ridout (7) and those of the group at Ann Arbor (3, 4).

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[4] Obtained from the Research Laboratories of the Eastman Kodak Company, Rochester, New York.

Best and Ridout (7) showed that casein (when fed as a 30 per cent supplement to a basal diet containing 5 per cent beef muscle powder[5] as the protein) exerted a greater lipotropic effect than was obtained with equivalent amounts of methionine and cystine. They found that a 16 per cent casein supplement, which provided approximately 49 mg. of methionine per rat per day, exerted about the same lipotropic effect as a 0.5 per cent methionine supplement which provided 47 mg. They noted, however, that at lower or higher intakes of the two substances the effects were by no means identical.

Tucker, Treadwell, and Eckstein (3) used a basal ration containing 5 per cent casein which was supplemented in one series with casein and in another with amounts of methionine and cystine equivalent to that contained in the protein supplement. They concluded that the lipotropic action of casein could be explained on the basis of the methionine and cystine contents of the diets. These workers used casein as the only protein, and at levels of 15 and 20 per cent, a range in which Best and Ridout had also found approximately equivalent lipotropic effects from free and bound methionine.

It has been reported (5, 7, 20, 21) that the lipotropic action of methionine differs from that of casein in that, although relatively small doses produce a significant fall in liver fat, further increases in the methionine supplement do not cause any further decrease in spite of the fact that 10 to 16 per cent (3 to 4 times the normal) fat is still present in the liver. Adequate amounts of casein, on the other hand, reduce the liver fat further (5 to 8 per cent). It appears, therefore, that methionine requires some other constituent of casein to exert its full lipotropic effect. In spite of these findings, Treadwell, Groothuis, and Eckstein (4) maintain that the lipotropic action of methionine as the free amino acid is superior to that of an equivalent amount of methionine in the form of the protein, casein.

The data in the present paper appear to offer some explanation for the different results obtained in assessing the magnitude of the part played by methionine in accounting for the lipotropic action of casein. The data obtained from Diets 2 and 3 (Series I, II, and III) and Diets 5 and 6 (Series II) appeared to support the views of Eckstein and his collaborators. However, exact equivalence of methionine in the pairs of diets was not achieved; the diets supplemented with free methionine contained 79 mg. per 100 gm. of food excess of the free amino acid. The greater lipotropic action of these diets *might* therefore be accounted for by this slight excess of methionine. That this is not so, however, can be easily shown. In Table I, diets containing amounts of methionine equivalent to those inadvertently fed in Diets 3 and 6 may be found. Data for such a comparison are presented in Table IV.

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[5] This beef powder contains 2.90 per cent methionine and 0.89 per cent cystine (3.19 and 0.97, respectively, corrected for moisture and ash) as determined by Kassell and Brand's (12) modification of Baernstein's method (13).

A comparison of data from Diet 3 of Series I, II, and III with those from Diet A-10, which provides about the same amount of methionine, reveals that the former exerts a slightly greater lipotropic effect. Whether or not these differences are great enough to be of significance may be questioned. No doubt can be entertained, however, as to the significance of the difference between the data from Diet 6, Series II, and the data from Diets A-7 and B-7. Here free methionine is markedly more lipotropic than an equivalent amount fed as casein.

TABLE IV

Effect of Dietary Methionine on Liver Fat

Source of data	Protein	Total methionine in diet	Liver fat
		mg. per 100 gm.	*per cent liver weight*
Diet 2, Series I	Casein + gelatin	376	34.1
" 2, " II	" + "	376	20.3
" 2, " III	" + "	376	30.8
" 3, " I	Gelatin + methionine	455	14.4
" 3, " II	" + "	455	16.4
" 3, " III	" + "	455	15.0
" A-10	Casein + gelatin	462	18.5
" 5, Series II	" + "	290	28.9
" 6, " "	Gelatin + methionine	329	16.9
" A-7	Casein + gelatin	333	26.5
" B-7	" + "	333	27.4

A consideration of the liver fat levels obtained by feeding Diet 4 of Series I, II, and III (Tables II and III) shows that any superior lipotropic efficacy of free over bound methionine has been completely obliterated by the inclusion of the essential amino acids. This effect is brought about presumably by improving the nutritional value of the diet.

The casein-supplemented diets used by Tucker *et al.* (3) and Treadwell *et al.* (4) differed from the diets supplemented with methionine + cystine by the amount of non-sulfur-containing amino acids in the casein supplement. Hence their diets differed as did our Diets 2 and 3 (Series I, II, III). The attempt to equalize the total N and essential amino acid intakes (Diet 4, Series I, II, III) threw a new light on the whole problem. Their casein-supplemented diets, being more nearly nutritionally complete, would be expected to lead to higher liver fat levels (*cf.* Griffith *et al.* (17–19)). The rats on these diets ate more and gained more, as Treadwell *et al.* mention. They pointed out that the apparently greater lipotropic effect of free methionine might be explained by larger amounts of methionine required for the synthesis of tissue protein in the casein-fed group, creating a decrease in the amount available for lipotropic purposes and thus leading to higher liver fat levels. The experimental data reported above give strong support to this explanation of their findings.

It is possible that the results obtained by Best and Ridout (7) when feeding high casein diets may be explained by the fact that such diets supplied not only adequate amounts of growth essentials but apparently also an excess of methionine which in the presence of these essential metabolites was able to exert maximal lipotropic action. The possibility cannot be dismissed, however, of the presence in casein of other amino acids or combinations of amino acids having lipotropic action.

As mentioned above, the evidence available to date indicates that free methionine, under the experimental conditions hitherto used, has a limited lipotropic effect in so far as it will not lower liver fat to the same extent as does an adequate amount of casein. However, the lipotropic effect of free methionine when added to a basal diet containing sufficient of the essential amino acids to permit maximal growth *may* equal that of an equivalent amount of casein. Experiments along these lines, covering a wide range of dietary casein, are already under way in this laboratory and preliminary results confirm the findings reported above and also those of Best and Ridout (7). The discrepancy between the conclusions of the latter workers and those of the Ann Arbor group appears to be due to the different protein levels of the diets used. The conclusions reached and explanations offered in the present paper appear to be substantiated by these latest studies, details of which will be published shortly.

The writers are deeply indebted to Professor C. H. Best for valuable criticism and advice during the preparation of this paper. The work was supported in part by a grant from the Banting Research Foundation to the Banting and Best Department of Medical Research.

SUMMARY

1. It has been shown that certain essential amino acids have a marked effect on the lipotropic efficacy of methionine.

2. No significant difference was noted in the lipotropic effect exerted by methionine when fed as the free amino acid or in casein, provided the essential amino acids were approximately equalized in the two diets.

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WATER, NITROGEN, AND ELECTROLYTE CONCENTRATION IN BRAIN

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(Received for publication, February 26, 1944)

Chemical and functional changes in the organs of the body usually are attributed to the tissue components. If this is a correct conception, not only the composition and functional capacities of all of the tissue phases should be known but also the quantitative measurements of the relative amounts of these phases. Stimulated by these possibilities, numerous workers in the past few years have intensively studied the partition of electrolytes in mammalian tissue. Results of research on muscle, liver, kidney, and skin of the dog having already been presented, the defining of values for the distribution of water, nitrogen, and electrolytes in brain was the aim of this project. Utilization of these data for a comparison of the right and left cerebral hemispheres and of the hemisphere and the cerebellum also was planned. These findings would provide normal mean values for further investigative study of brain.

There have been few histochemical studies on brain tissues. Tupikova and Gerard (1) published an abstract in 1937 on the salt content of neural structures; Yannet and Darrow in 1938 (2) contrasted the composition of skeletal muscle, liver, and brain in two groups of cats, a young group and an older group. Stewart-Wallace (3) in 1939 presented water and electrolyte data on human brain tissue obtained at autopsy. Manery and Hastings (4) in 1939 published data on brain from rats and rabbits.

The present study includes analytical data on brains from twenty-two normal dogs apportioned as follows: five normal animals in each of which the right and left cerebral hemispheres were analyzed separately; five normal animals from which portions of fresh brain and ether-extracted portions from the same brain were analyzed; and twelve normal dogs from which serum, hemispheres, and cerebellums were analyzed.

Procedures and Methods

The normal dogs were kept under observation in metabolism cages and maintained on an alternate meat and dog chow[1] diet for at least 3 to 4 weeks. When used they were in excellent physical condition. They were grown dogs of unknown age.

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[1] Globe dog chow Blox.

Blood was drawn under oil from the femoral artery and allowed to clot. The serum was used for the analyses of pH, total CO_2, chloride, water, sodium, potassium, calcium, magnesium, and total nitrogen.

Immediately after withdrawal of blood, the brain was removed. It was our purpose to effect removal of the brain as soon after death as possible. All craniotomies were performed under nembutal anesthesia. In nine of the animals, the muscle and most of the bone were removed from the skull. Invariably this procedure resulted in moderate to severe loss of blood and a gradual drop in blood pressure over a period of about 30 minutes. Toward the end of the operation there was usually massive bleeding during the suboccipital exposure. In this group of dogs the brain was completely removed in from 2 to 20 minutes after death. Three dogs under nembutal anesthesia were killed by air embolism. In these the brain was removed within 11 to 14 minutes after death. The brain was then wiped quickly with gauze to remove adherent blood. The brain stem and cerebellum were separated by section through the peduncles at the level of the superior colliculi and the cerebral hemispheres divided in the sagittal plane.

Each hemisphere and the cerebellum were placed in glass-stoppered weighing bottles and allowed to come to room temperature. Each hemisphere was weighed for total mass and then finely minced with scissors. Weighed aliquots were used for all analyses. The procedure developed for weighing the aliquots of wet brain was to place and weigh a filter paper (4.25 cm.) on a small watch-glass, then transfer a sample of wet minced brain to the paper from the weighing bottle with a stainless steel spatula, weigh again, and then drop the paper with contents into the chloride tube or the Kjeldahl flask. The minced tissue was weighed directly into the silica beakers for the sodium and potassium determinations and into the platinum dishes for the calcium and magnesium determinations.

The cerebellum with the brain stem was treated exactly in the same manner.

The following determinations were made on the hemispheres and cerebellum: water, chloride, sodium, potassium, calcium, magnesium, and total nitrogen. The chemical methods for these determinations were the same as those previously reported (5, 6). The approximate amounts of the wet brain used for the different determinations were as follows: chloride 1.3 gm., total nitrogen 0.5 gm., sodium and potassium 8 gm., and calcium and magnesium 10 gm.

Results

Regrettably, the analytical results could not be expressed on the basis of fat-free tissue because the organic solvents used for the extraction of the free fat from the tissue also extracted large (and constant) amounts of other brain lipids and electrolytes. These findings are shown in Tables I and II and were obtained by the following procedures. The hemisphere and cerebellum were each placed in a large flat bottom glass-stoppered weighing bottle, minced with the tip of scissors, and dried to constant weight in a 102° oven for 48 hours. The dried tissues were first extracted with ether and then with low boiling petroleum ether (5). The tissues were dried in the oven again for an hour to remove organic solvents and weighed. The tissue residue was smashed to a fine powder in the special apparatus described in a previous work (5). Aliquots of the smashed tissue were then weighed on small filter papers (4.25 cm.) for the chloride and nitrogen analyses and placed directly in silica beakers and platinum dishes for the sodium and potassium and the calcium and magnesium analyses, respectively. The data so obtained are presented in Table I.

TABLE I

Water and Electrolyte Content of Brain Expressed in Units per Kilo after Ether Extraction

Dog No.		H_2O	Fat extracted	Total N	Cl	Na	K	Ca	Mg
		gm.	*gm.*	*gm.*	*mM*	*mM*	*mM*	*mM*	*mM*
B_1	Right hemisphere	857.2	104.3	18.76	33.19	35.36	70.7		
	Left "	856.1	104.3	18.42	34.03	35.20	70.5		
	Cerebellum	856.4	120.8	19.00	32.70				
B_2	Right hemisphere	855.4	109.5	18.41	34.80	34.86	68.9	1.09	4.61
	Left "	856.3	109.4	18.69	34.30	34.16	70.4	1.22	4.90
	Cerebellum	853.5	131.7	18.85	30.36	31.50	62.7		
B_3	Right hemisphere	857.4	100.7	18.22	35.54	35.08	72.5		
	Left "	857.5	101.2	18.32	35.59	36.26	74.7	1.10	4.91
	Cerebellum	854.0	118.4	18.73	33.60	33.76	67.3		
B_4	Right hemisphere	857.8	118.8	18.58	37.60	29.00	66.4	1.02	5.09
	Left "	858.0	118.7	18.89	36.26			0.98	4.52
	Cerebellum	854.5	138.5	18.79	30.56	30.66	61.7		
B_5	Right hemisphere	851.5	106.5	18.69	35.33	33.26	69.0	1.07	4.77
	Left "	851.0	106.0	18.88	34.98	33.44	69.3	1.09	5.09
	Cerebellum	845.7	124.8	19.24	33.14	33.06	65.6		

It became apparent at once that the ether and petroleum ether were extracting some (constant amount) of the lipids. Whether the lipids were taking into solution any of the electrolytes had to be ascertained. Instead of analyzing the ether extracts, which is virtually impossible to do because of the high concentration of lipids in solution and the loss of ether by evaporation, we carried out analyses on portions of the same fresh brain both with and without ether extraction. Table II gives the results of these determinations. It is clear, therefore, that after ether extraction the concentration of electrolytes in the brain tissue was changed. The organic solvents, ether and petroleum ether, had removed some of the brain lipids which carried with them large amounts of chloride, sodium, potassium, and magnesium and small amounts of nitrogen and calcium. These results are somewhat in keeping with the findings of Christensen (7) who showed the dissolving power of petroleum ether solutions of phospholipids from erythrocytes and plasma for alkali halides.

Whether the constant amounts of electrolytes removed are the ones associated with myelin or whether they represent a physical phenomenon cannot be stated at this time. These results prove that this method is not satisfactory for removing only the free neutral fat from brain tissue for electrolyte analysis. Therefore, the amount of free fat was not determined. Since the total water content and consequently the total tissue solids of brain were found to be constant, the free fat content must be constant in value. Furthermore, it is well known histologically that the amount of free neutral fat in normal brain is low.

TABLE II

Comparison of Concentrations of Electrolytes in Fresh Hemisphere and the Hemispheres after Extraction with Ether

Dog No.		Cl	Na	K	Ca	Mg	Total N
		mM	*mM*	*mM*	*mM*	*mM*	*gm.*
SS_4	Hemisphere*	35.85	50.8	98.0			18.7
	" (ether)†	28.00	28.7	53.3			15.9
SS_5	"	36.92	51.1	88.1	1.23	5.23	18.5
	" (ether)	27.82	30.0	51.0	1.00	4.27	15.8
SS_6	"	35.70	51.8	88.6	1.05	5.63	18.5
	" (ether)	28.55	30.9	55.4	1.01	4.47	16.2

* The units are expressed per kilo of fresh hemisphere.

† The units are expressed per kilo of fresh hemisphere after extraction with ether.

Values for Right and Left Hemispheres—The results of the analyses of the right and left hemispheres from three of the five dogs are given in Table III. It will be observed that the total mass, water, nitrogen, and electrolyte concentrations in the right and left hemispheres of the same animal were approximately the same. This finding is not surprising, for similar results have been found with other right and left tissues. The analyses of right and left kidneys (5) or right and left testes, etc., give the same total mass and electrolyte concentrations. These data indicate that the right and left hemispheres of normal brain are alike in water, nitrogen, and electrolyte distribution and therefore it is not necessary to separate them for

TABLE III

Water and Electrolyte Content of Normal Right and Left Hemispheres of Brain

The values are given in units per kilo.

Dog No.	Hemisphere	Total weight	H_2O	Cl	Na	K	Ca	Mg	Total N
		gm.	*gm.*	*mM*	*mM*	*mM*	*mM*	*mM*	*gm.*
B_6	Right	6.604	760.5	36.52	48.1	98.2	0.97	4.98	20.1
	Left	5.779	761.0	36.39	47.7	101.2	0.92	5.04	19.4
B_{10}	Right	6.434	767.0	36.05	50.6	92.4	1.12	6.59	19.0
	Left	4.518	764.0	36.15	51.7	94.6	0.93	6.07	18.3
B_{11}	Right	6.997	763.1	36.47	51.5	97.5	1.10	6.33	18.5
	Left	7.136	760.8	37.22	51.8	97.7	1.18	6.43	18.6

TABLE IV

Analyses of Serum and Brain from Normal Dogs

The values are given in units per kilo.

Dog No.		H_2O	Cl	Na	K	Ca	Mg	Total N
		gm.	*mM*	*mM*	*mM*	*mM*	*mM*	*gm.*
B_6	Serum	921.5	109.8	140.5	4.80	2.73	0.87	
	Hemisphere	760.5	36.52	48.1	98.2	0.97	4.98	20.1
	Cerebellum	748.2	34.56	48.1	95.6			20.0
B_8	Serum	924.6	111.3	143.0	4.61	2.19	0.88	
	Hemisphere	748.3	35.24	47.3	90.1	1.10	6.00	19.5
	Cerebellum	731.8	34.54	50.7	96.6			19.5
B_{10}	Serum	920.4	110.8	146.7	4.35	2.59	0.92	
	Hemisphere	767.0	36.05	50.6	92.4	1.12	6.59	19.0
	Cerebellum	749.0	33.90	52.1	94.9			19.2
B_{11}	Serum	912.2	108.7	143.8	4.82	2.43	0.93	
	Hemisphere	763.1	36.47	51.5	97.5	1.10	6.33	18.5
	Cerebellum	743.3	35.61	51.9	94.7			18.4
SS_6	Serum	926.8	110.8	139.0	4.57	2.41	0.94	
	Hemisphere	754.2	35.70	51.8	88.6	1.05	5.63	18.5
	Cerebellum	748.3				1.19	5.22	
SS_7	Serum	934.4	112.7	142.7	4.52	2.36	0.85	
	Hemisphere	770.8	37.70	51.8	101.8	1.05	5.62	18.9
	Cerebellum	747.8				1.02	5.82	
Mean of 12 dogs	Serum	923.6	108.8	141.4	4.66	2.47	0.95	
	σ	6.4	4.4	2.8	0.24	0.16	0.08	
	Hemisphere	761.3	36.71	51.0	95.6	1.07	5.63	18.9
	σ	8.3	1.05	2.4	4.7	0.07	0.56	0.3
	Cerebellum	745.0	35.19	50.8	92.7	1.07	5.40	19.1
	σ	7.0	0.89	1.7	4.0	0.07	0.30	0.5

chemical analyses, though separate analyses are useful as reciprocal controls.

Values for Normal Hemispheres and Cerebellums—The analytical data of six representative normal whole hemispheres, cerebellums, and serums are given in Table IV, together with the mean values with standard deviations for twelve normal brains. The water and nitrogen content and the electrolyte concentrations from all animals were the most constant of any tissues studied thus far. The standard deviations were small in all determinations of the twelve brains.

Because the water content of both the hemispheres and the cerebellums was lower than the values reported by other investigators on mammalian brain, several determinations made by our standard method of drying the minced tissue for 48 hours at 102° were checked by drying the tissue for 96 hours. The results were no different. Determinations also were made on weighed minced aliquots of tissue stirred with acetone to disperse the brain particles. The acetone was then evaporated from the mixture and the contents dried at 102° for 48 hours. Again the results were unchanged.

It will be noted that in brain, as in other tissues, potassium represented the larger part of the univalent base and magnesium nearly all of the bivalent base. The calcium value, 1.07 mM, ±0.07, was higher than anticipated. In skeletal muscle and liver, the values reported were 0.82 and 0.83 mM, respectively, and since these amounts of calcium could not be allocated entirely in an ionized form to the interstitial fluid it was assumed that the calcium was combined with the connective tissue fibers in the extracellular phase. In brain, the high concentration of calcium further indicates that all of the tissue calcium in the interstitial fluid is not ionized.

DISCUSSION

If the structure of all the tissues of the body were simple enough to be divided into two phases, extracellular and intracellular, the relative volumes could be calculated from the serum and tissue composition as was done for skeletal muscle by Hastings and Eichelberger (8). Brain, however, is a very complex tissue comprised of many different types of cells with variation in structure and function and for that reason cannot be treated in the same manner as the simple tissue, skeletal muscle.

That brain tissue reacts differently than the other tissues of the body has been demonstrated by numerous investigators. In 1937, Wallace and Brodie (9) showed that intravenously injected iodides, bromides, or thiocyanates did not enter the spinal fluid nor the brain tissue at the same rate or to the same extent as they were distributed from the plasma into the other tissues. In 1938, Amberson *et al.* (10) demonstrated in their perfusion experiments that the retention of chloride in nervous tissue varied from other tissues. Even when plasma chlorides had been greatly reduced in concentration and as a consequence all tissue chlorides were reduced, brain retained most of its chloride. In 1941, Manery and coworkers (11, 12), working with injected radioactive sodium and chloride, concluded that the extent and rate of penetration of these isotopes into tissues indicated with few exceptions the tissues in which these ions were all extracellular. Brain was one of the exceptions. They found that neither radioactive sodium nor chloride entered the brain for long periods of time after injection. They could not decide whether this delayed entrance was because some of the sodium and chloride was intracellular or because there was a barrier between the blood and the extracellular phase of the brain.

In simple tissue, muscle, the total solid of the tissue represents mostly the protein of the tissue cells. In brain the total solids represent not only the protein fraction and the lipid fraction of the cells, but also the solid of the extracellular phase. In brain there is only about 0.3 per cent of connective tissue (blood vessels) as determined by Lowry, Gilligan, and Katersky (13), but there is present a complicated substance, myelin. This contains organic solids of lipids rich in nitrogen and phosphorus and also the possibility of some extracellular fluid (1). If this fluid is associated with the myelin, the extracellular phase of brain cannot be assumed to be a single aqueous phase. Quantitative interpretation of the analytical data for brain into exact volumes of extra- and intracellular phases consequently cannot be made at this time. It is possible, nevertheless, to make some tentative conjectures.

The concentration of ions in a tissue generally indicates the size of the phases in the tissue. In muscle, for example, the chloride and sodium are confined to the extracellular phase, and potassium to the tissue cells. In the complex tissue of brain the high concentrations of sodium and chloride would indicate a large extracellular phase and, if all were extracellular the calculated extracellular phase from the experimental chloride and sodium would be 30 and 35 per cent, respectively. Contrariwise, if all of the sodium and chloride of brain were extracellular and existed as an ultrafiltrate of the serum, the ratio of sodium to chloride in this phase should approximate the serum value of 1.26. Instead, a ratio of 1.4 was found, indicating that all of the sodium and chloride in brain is not extracellular and that there must be some cells in which the intracellular sodium exceeds the intracellular chloride. This finding may be the result of the presence of myelin, whose definite composition is not known. It is known histologically that the myelin sheaths surround the axones, but it has not been determined whether or not these myelin sheaths are associated with an aqueous phase having a high concentration of sodium and chloride similar to that of the peripheral nerves of which the majority of fibers is myelinated (1, 10). From the evidence it is reasonable to assume that it does.

The potassium values of 95.6 mM per kilo of hemisphere and 92.7 mM per kilo of cerebellum are not different from those found in skeletal muscle (97.1 mM). This suggests that the intracellular phase of brain is similar to that of muscle if the concentration of this basic univalent ion is indicative of the size of the intracellular phase of a tissue. At the same time, the concentration of magnesium was found to be 5.5 mM per kilo, which is about one-half that found in skeletal muscle (9.05 mM). These discordant parallelisms make quantitative interpretation of the data unsatisfactory.

Separation of the brain into the cerebral hemispheres and cerebellum showed that the cerebellum as compared with the hemispheres was lower in water and chloride. This denotes that the extracellular phase of the cerebellum is slightly lower than that of the hemispheres. Despite the complexity of brain tissue the data for each part of the brain are most constant in their consistency. These results may prove useful for further experimental work on brain as control data, in view of the fact that it is impractical to take control brain tissue from the experimental animal.

SUMMARY

1. Procedures are presented for water and electrolyte analyses of cerebral hemispheres and cerebellum.

2. Total water, nitrogen, and electrolyte concentrations were determined in brain, which was removed by bilateral craniotomy from normal dogs. For analyses the brain was separated into the cerebral hemispheres and cerebellum with the brain stem.

3. The right and left hemispheres from the same animal gave the same analytical results. The hemispheres gave the following mean average results which are expressed as units per kilo of hemisphere: total water 761.3 gm., σ ±8.3; chloride 36.71 mM, σ ±1.05; sodium 51.0 mM, σ ±2.4; potassium 95.6 mM, σ ±4.7; calcium 1.07 mM, σ ±0.07; magnesium 5.63 mM, σ ±0.56; and total nitrogen 18.9 gm., σ ±0.3. The cerebellum with brain stem gave the following mean average results: total water 745.0 gm., σ ±7.0; chloride 35.19 mM, σ ±0.89; sodium 50.8 mM, σ ±1.7; potassium 92.7 mM, σ ±4.0; calcium 1.07 mM, σ ±0.07; magnesium 5.40 mM, σ ±0.30; and total nitrogen 19.1 gm., σ ±0.5.

4. Because the analyses of the hemispheres and the cerebellum following extraction of the dried tissue with ether and petroleum ether gave low concentrations of chloride, sodium, and potassium, the analytical results were not expressed in terms of fat-free tissue.

5. The analytical results are of value for further experimental work on brain as control data, since it is impractical to take control brain tissue from the experimental animal.

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PRODUCTION OF RIBOFLAVIN DEFICIENCY WITH PHENAZINE ANALOGUES OF RIBOFLAVIN

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(Received for publication, March 18, 1944)

Since it was shown recently in this laboratory (1) that the growth-inhibiting properties of benzimidazole were overcome by the aminopurines, adenine and guanine, it was considered of interest to determine whether the structural change involved in passing from the purines to benzimidazole was one of general applicability for the production of inhibitory compounds. Benzimidazole is derived from purine by substitution of the 2 N atoms in the pyrimidine ring by C atoms. In a consideration of vitamins and similar metabolites to which this structural change might be applied, it was seen how the pyrimidine ring in riboflavin might be exchanged for a benzene ring to bring about an analogous change. The resulting compound would be a substituted phenazine.

Several attempts were made to synthesize the required dihydroxydimethylribityldihydrophenazine but without success. Impure preparations of the related unhydroxylated compound were obtained and found inhibitory of growth of certain bacteria. However, the effects were not reversed by increased amounts of riboflavin, and hence were difficult to interpret.

A method for the synthesis of 2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine was then devised. This substance proved capable of producing deficiencies of riboflavin in both animals and microorganisms. The synthesis was based on the methods of Kehrmann and Messinger (2) for the preparation of 2,4-dinitro-10-alkyldihydrophenazines. 1-Ribitylamino-2-amino-4,5-dimethylbenzene reacted with picryl chloride to yield the substituted diphenylamine (I). This was converted smoothly and easily to 2,4-dinitro-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine (II) which was then reduced to the desired amine (III). The structural similarity to riboflavin (IV) is apparent.

The diaminophenazine caused inhibition of the growth of certain bacteria, and this inhibition was removed by additional amounts of riboflavin. With one exception the organisms which were prevented from growing required riboflavin as a growth factor, and those species which were not affected by similar quantities of the phenazine were not stimulated in growth by riboflavin. However, a sufficiently large number of species and a

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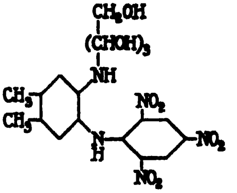
* With the technical assistance of J. Backstrom and J. Clifford.



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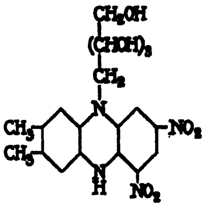

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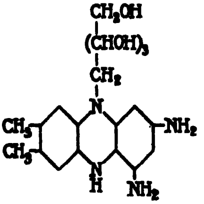

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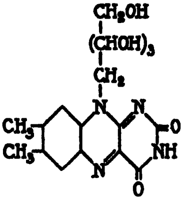

IV

wide enough range of concentration of the phenazine were not used to make sweeping generalizations on this point. Because of the instability of the diaminophenazine when exposed to air, it was found advisable to use the dinitrophenazine and to reduce it in the culture medium by heating with finely divided iron. Comparative tests with two species showed that when the dinitrophenazine was reduced in this manner it had a similar effect to that of the pure diaminophenazine. The dinitrophenazine itself was not inhibitory of the growth of bacteria.

The dinitrophenazine produced mild riboflavin deficiency when it was fed to mice. This condition was prevented by the feeding of large amounts of riboflavin. The deficiency usually did not prove fatal, even with considerable amounts of the phenazine, and hence was not as dramatic as in the case of thiamine deficiency produced by pyrithiamine (3).

The production of riboflavin deficiency by the phenazines together with the observations on benzimidazole (1) makes it seem probable that if benzene rings are substituted for pyrimidine rings in other biologically active compounds it may be possible to produce drugs whose effects are in part traceable to the functional elimination of these biologically active compounds from certain processes. At present, the only other general method of producing inhibitory compounds is the substitution of sulfonic acid or amide groups for carboxyl groups as in the case of the sulfonamides or thiopanic acid (4–6).

During the preparation of this manuscript a paper appeared (7) which described the production of riboflavin deficiency in several bacteria by the use of 6,7-dichloro-9-ribitylisoalloxazine. This compound was the same as riboflavin except that the methyl groups had been replaced by Cl atoms. The alteration in the vitamin molecule was therefore fundamentally different from that produced in the present work.

EXPERIMENTAL

2-Ribitylamino-4,5-dimethylphenyl-2′,4′,6′-trinitrophenylamine—2.7 gm. of 1-ribitylamino-2-amino-4,5-dimethylbenzene[1] and 1.4 gm. of sodium acetate were dissolved in 40 cc. of 50 per cent alcohol, and to the solution were slowly added 2.6 gm. of picryl chloride dissolved in 60 cc. of alcohol. After the two solutions were mixed, the brown suspension which resulted was shaken vigorously for an hour, and placed at 4° overnight. The brown crystals were filtered off and washed thoroughly with 50 per cent alcohol, and dried *in vacuo*. Yield, 4.6 gm. It was not possible to purify the substance because of the ease with which it was converted into the dinitrophenazine. Thus, recrystallization from alcohol yielded only the purple needles of the phenazine; m.p. 216–218°, with decomposition.

2,4-Dinitro-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine—4.6 gm. of the above diphenylamine, 6 gm. of sodium acetate, and 200 cc. of alcohol were heated to boiling for 3 hours. The brown color of the starting material gradually changed to a deep bluish purple. After the reaction mixture had stood overnight at 4°, the crystals were filtered off and washed thoroughly with alcohol, water, and alcohol. They were well formed deep bluish purple needles. They were purified by recrystallization from ethyl benzoate. Yield, 4.0 gm.; m.p. 218–220°, with decomposition.

$C_{19}H_{22}O_8N_4$. Calculated, C 52.5, H 5.1; found, C 51.5, H 5.3

Satisfactory analyses for N were not obtained since determinations on the same material gave values ranging from 8 to 11 per cent. The compound was insoluble in water and in many organic solvents, but was relatively soluble in acetic acid or ethyl benzoate. It dissolved in 20 per cent HCl. The addition of $SnCl_2$ to the solution in 20 per cent HCl caused the color to change from purple to green.

2,4-Diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine Hydrochloride—1 gm. of the above dinitrophenazine was stirred into 25 cc. of 20 per cent HCl, and then 2 gm. of powdered Sn were added. Stirring was continued for 1 hour, and then the mixture was refluxed for an hour. Tin was next removed by passing H_2S into the solution and filtering off the precipitate. The yellow filtrate so obtained was concentrated under reduced pressure to dryness. The residue was dissolved in the minimal amount of hot alcohol. The solution was filtered, and half a volume of ether was added to the hot filtrate. In this manner 750 mg. of yellow-brown crystals were obtained. These crystals became very dark when exposed to air.

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[1] We wish to thank Dr. H. M. Wuest of Hoffmann-La Roche, Inc., for generous gifts of this compound, and Dr. G. M. Shull of Chas. Pfizer and Company, Inc., for a gift of the azo derivative of this amine.

$C_{19}H_{26}N_4O_4 \cdot HCl$. Calculated. C 55.6, H 6.6, N 13.7
Found. " 54.9, " 6.5, " 13.7

The compound was readily soluble in water and moderately soluble in cold alcohol. When the compound which had darkened from exposure to air was dissolved in water, the solution appeared red-brown. This color was reduced to yellow by a crystal of $Na_2S_2O_4$. When the yellow solution so obtained was shaken in air, the color quickly deepened. The final color was more brown and less red than the original.

Method of Bacterial Test—The riboflavin-free medium of Landy and Dicken (8) was used throughout this work. For *Staphylococcus aureus* and hemolytic streptococcus H69D it was diluted with an equal volume of water. Varying amounts of riboflavin and of the phenazine to be tested were added to 10 cc. portions of the medium contained in 20 mm. test-tubes. The tubes were sterilized by heating in an autoclave for 15 minutes at 15 pounds pressure, cooled, and inoculated with 1 drop per tube of a suspension of the cells of the desired organism. The cells for the inoculum were washed three times and suspended in a volume of sterile buffer 50 times that of the original culture. The tubes were incubated at 37°, or at 30° for *Streptococcus lactis* R and *Lactobacillus arabinosus*, until maximal growth was observed in the control tubes which contained riboflavin as the only addendum (40 to 70 hours). The pH of the contents of each tube was then determined with a glass electrode. Because of the deep color of the phenazines it was not possible to make comparisons of turbidity. A curve was constructed which related pH to the quantity of phenazine added in the presence of 0.03 γ of riboflavin per cc., and from this curve the amount of phenazine required to cause half the maximal effect in the presence of this amount of riboflavin was determined. This value served as a basis for comparison of the various compounds and organisms.

Whenever the dinitrophenazine was to be reduced in the culture media, finely divided iron (reduced iron) was added to the media before the tubes were autoclaved. The reduction apparently occurred during autoclaving. Approximately 4 mg. of Fe per tube were used. When the diaminophenazine was sterilized by filtration and added to the tubes after they were autoclaved, it was found that no greater potency was observed than when the amine was added before autoclaving, and hence it was concluded that heat did not harm the compound under the conditions studied.

Inhibition of Growth of Lactobacillus casei and Its Reversal by Riboflavin—

In Table I is shown the effect of various amounts of riboflavin and of the dinitrophenazine, the dinitrophenazine reduced in the tubes with Fe, and the diaminophenazine on the growth of *Lactobacillus casei* as measured by the pH of the cultures. It can be seen that the reduced dinitrophenazine and the diaminophenazine had potencies of the same order of magnitude for inhibition of growth, and that the dinitrophenazine was ineffective. Furthermore it can be seen that sufficient amounts of riboflavin completely abolished the effect of the phenazines.

TABLE I

Effect of 2,4-Dinitro-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine and Its Reduction Products on Growth of Lactobacillus casei in Presence of Varying Amounts of Riboflavin

Riboflavin	Dinitrophenazine	Reduced dinitrophenazine	Diaminophenazine	pH of culture
γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	
0.0	0	0	0	6.9
0.03	0	0	0	4.0
100.0	0	0	0	4.0
0.03	0	1000	0	7.1
0.03	0	200	0	7.0
0.03	0	100	0	6.2
0.03	0	60	0	5.0
0.03	0	50	0	4.4
0.15	0	100	0	4.1
100.0	0	200	0	4.3
0.03	1000	0	0	4.1
0.03	0	0	400	7.1
0.03	0	0	100	5.4
0.03	0	0	50	4.3
100.0	0	0	300	4.3

Effect of Phenazines on Other Bacteria—In Table II are shown the amounts of the various preparations required for half maximal inhibition of growth of various bacterial species in the presence of 0.03 γ of riboflavin per cc. In each of the cases in which inhibition of growth occurred, the effect was abolished by additional amounts of riboflavin. The requirement of the various species for riboflavin as a growth factor is also listed, and it can be seen that with the exception of *Lactobacillus arabinosus*, only those which required riboflavin were affected by the phenazines.

Production of Riboflavin Deficiency of Mice with Dinitrophenazine—Weanling mice (9 to 11 gm.) were caged individually on screen floors and fed a ration composed of sucrose 75 gm., casein (Labco) 18 gm., salts (9) 5 gm., fortified corn oil (10) 1 gm., thiamine 0.2 mg., pyridoxine 0.2 mg., calcium pantothenate 2 mg., nicotinic acid 10 mg., choline 100 mg., and inositol 100 mg. This diet, plus riboflavin, has been shown to be adequate for mice (11). The basal control animals received the ration plus riboflavin. The test mice were fed this basal ration supplemented with varying amounts of the dinitrophenazine, and others received the basal ration plus the dinitrophenazine and increased riboflavin. Finally, some mice received the basal ration and in addition a solution of the diaminophenazine administered orally once daily. The results are summarized in Table III. It can be seen that the animals which received the dinitrophenazine grew

TABLE II

Relative Effectiveness of 2,4-Dinitro-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine, Its Reduction Product Obtained with Reduced Iron, and Corresponding Pure Diaminophenazine in Inhibition of Growth of Various Bacteria

Organism	Amount required for one-half maximal inhibition			Riboflavin
	Dinitrophenazine	Reduced dinitrophenazine	Diaminophenazine	
	γ per cc.	*γ per cc.*	*γ per cc.*	
Lactobacillus casei	No effect with 1000	80	110	Required
Hemolytic streptococcus H69D		330		"
Lactobacillus arabinosus	No effect with 1000	330	160	Not required
Staphylococcus aureus		No effect with 1000		" "
Streptococcus lactis R		" "		" "
Escherichia coli		" "		" "

TABLE III

Effect of 2,4-Dinitro-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine and Corresponding Diaminophenazine on Growth and Survival of Mice

Riboflavin	Dinitrophenazine	Diaminophenazine	No. of mice	No of deaths	Average gain
mg. per 100 gm. ration	*mg. per 100 gm. ration*	*mg. per day*			*gm. per wk.*
0.08	0	0	7	0	2.3
0.10	0	0	9	0	3.3
100.0	0	0	3	0	3.5
0.08	100	0	3	0	1.6
0.10	200	0	8	1	2.8
5.0	200	0	6	0	2.7
100.0	200	0	9	0	4.2
0.08	0	1.0	3	0	2.1

at a reduced rate, while those which had the dinitrophenazine and the additional riboflavin in sufficient amounts grew normally. It is to be noted that large amounts of riboflavin were required to nullify the effect of the dinitrophenazine. The dinitrophenazine underwent some change in the body, because the urine of the mice which received it was green. The color was presumably due to a reduction product of the phenazine.

Signs of riboflavin deficiency other than reduced rate of growth consist of a greasy, unkempt fur, and hyperirritability. The mice which received the dinitrophenazine were distinctly unkempt, and some of them were very hyperirritable as compared to the controls. However, only an occasional animal died, and the signs were not as severe as those seen in acute deficiencies. Sufficient amounts of riboflavin prevented the appearance of signs of the deficiency.

SUMMARY

A structural analogue of riboflavin, 2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine, has been synthesized by a series of reactions and shown to produce riboflavin deficiency in bacteria, and the dinitrophenazine from which it was derived has been shown to produce mild riboflavin deficiency in mice. The effects of the compounds were overcome by sufficient amounts of riboflavin.

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STUDIES ON ANIMAL DIASTASES

VI. THE DETERMINATION OF DIASTASE (AMYLASE) IN BLOOD

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(Received for publication, February 14, 1944)

In 1917 Myers and Killiàn (1) described a method of estimating the diastatic activity of blood in which soluble starch was incubated with oxalated whole blood and the reducing sugar formed determined with the modification of the Lewis-Benedict picric acid technique described by Myers and Bailey (2).

Somogyi has criticized this method in a publication in this *Journal* (3). He stated:

> "The trouble in these methods consists in the lack of standardization of important factors such as hydrogen ion concentration and electrolyte content, which are known seriously to influence diastatic activity. Even the fundamental requirement of establishing proper relationships between enzyme activity and the amount of substrate is disregarded in some instances. Myers and Killian for example employ with 2 cc. of plasma 10 mg. of starch, a quantity which is far from sufficient. As Sherman and his associates have shown as early as 1910, a proportionality between enzyme activity and copper-reducing power prevails only when a very small fraction, no more than 5 to 6 per cent of the starch, is converted into reducing sugars (expressed as maltose). Myers observed with his method conversions up to 40 and 60 per cent, expressed as glucose, which in terms of maltose equals 60 to 90 per cent. These are excessively high values in view of the fact that 80 to 85 per cent is regarded as the upper limit of diastatic starch conversion (*Grenzdextrin*). Under such conditions no quantitative relationship can exist between reducing power and enzyme activity, and the results obtained are erroneous. The inadequacy of 10 mg. of starch as substrate is even more obvious in abnormal cases (acute pancreatitis) in which 2 cc. of human blood plasma are capable of producing as much as 50 mg. of reducing sugars ('maltose') in 15 minutes."

Somogyi (4, 5) has repeated these criticisms without presenting any experimental data to support them, and has not referred to a paper by Myers and Reid (6) in which some of these points were fully discussed. It will be evident from the discussion which follows that Somogyi's criticisms are erroneous, and further that Somogyi's own digestion mixture (3) suffers from the serious deficiency of being essentially unbuffered.

A satisfactory method for the determination of the diastatic activity of blood should be based on criteria that satisfy the following questions: (1) Should whole blood or serum (or plasma) be used? (2) What is the best substrate? (3) What is the optimum concentration of substrate? (4) What is the optimum pH range, and how can this be maintained? (5) What is the optimum NaCl concentration in the digestion mixture? (6) At what temperature and how long should the incubation be carried out? (7) How can the enzyme action best be stopped and the blood proteins precipitated? (8) How may the reducing sugar formed by the enzyme action best be determined? (9) Do the conditions selected give a linear relationship over the range of values usually encountered? (10) Are the values reported intelligible to one unfamiliar with the method employed?

These questions will be discussed and answered briefly with special reference to the Myers-Killian method and a modification of this method which is presented in the present report.

Observations and Discussion

Use of Whole Blood, Serum, or Plasma—At the time the Myers-Killian method was described it was customary to carry out all blood analyses on oxalated whole blood whenever possible. Recent studies of the distribution of diastase between cells and plasma by Margaret Read[1] in this laboratory and by Bernard and Rosen (7) have indicated that the diastase of whole blood is contained almost entirely in the plasma. Read has shown that the diastase of whole blood is approximately 60 per cent of that of serum or plasma. It would thus seem best to employ serum or plasma for the determination. The principal advantage in using serum is that variations in cell volume, and hence in the amount of plasma in a given volume of whole blood, are eliminated. We have studied cases of severe anemia and noted that whereas whole blood diastase appears to be somewhat elevated the values obtained with serum are quite normal. However, it should be emphasized that measurements on whole blood are quite adequate for most purposes and that diastase values on whole blood when compared with normal figures on whole blood reflect increases or decreases in diastase.

Selection of Substrate—If blood diastase does play any rôle in carbohydrate metabolism, the substrate involved is most likely glycogen; so that an ideal substrate for blood diastase determinations would be glycogen. However, since essentially the same results are obtained with soluble starch (1), it can be satisfactorily employed instead of glycogen. At the present time we employ Lintner's soluble starch (Merck) which is a readily available preparation. We have made several studies with both blood diastase and with duodenal contents (8), which indicate that different lots of this starch give comparable values and that the results agree with those obtained with Small's (9) soluble starch which we have employed in many of our studies (6, 10).

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[1] Read, M. R., unpublished observations.

Concentration of Substrate—In any enzyme measurement it is essential to provide an adequate excess of substrate, such that in the period of reaction the equilibrium is not attained. This point has recently been discussed by the authors (11). One of Somogyi's (3) principal criticisms of the Myers-Killian method is that an inadequacy of substrate is provided. A paper of Sherman, Kendall, and Clark (12) is cited by Somogyi in which he states that these authors consider that a proportionality between enzyme activity and copper-reducing power prevails only when no more than 5 or 6 per cent of the starch is converted into maltose. Careful study of the paper by Sherman *et al.* reveals that no such statement was made and furthermore there are no data presented by them to indicate how far the proportionality exists. On the other hand Myers and Reid (6) presented data (their Fig. 2) which indicated that the amount of reducing sugar was proportional to the enzyme concentration over a range of values which encompasses essentially all the figures which have ever been reported from the senior author's laboratory. We are well aware of the fact that when high values are encountered a reduced amount of blood must be used in the enzyme measurement in order for the values to have any significance. It so happens that in none of our studies did we encounter patients with pancreatic disease showing markedly elevated diastase values (13). Data were presented on two cases of carcinoma of the head of the pancreas in our first paper (1) and shortly thereafter determinations were made on four additional cases of carcinoma of the pancreas and three cases of pancreatitis, the highest reduction found being 4.8 mg., all the determinations being well inside the linear relationships of the method. We have carried out investigations of blood diastase in dogs and in all of these studies only 0.2 cc. of blood was used (6), since the amount of diastase in dog blood is very much greater than that in human blood. At the present time the principal clinical usefulness of determinations of blood or serum diastase appears to be in the diagnosis and prognosis of acute pancreatitis (11, 14). Since one expects to find markedly elevated figures for blood or serum diastase in this condition, we feel that in the revised method which we are describing in this paper 50 mg. of substrate are to be preferred to 10 mg. However, it is equally accurate to employ 10 mg. of substrate and to repeat with decreased amounts of serum those determinations in which more than 5 mg. of starch are converted to reducing sugar.

Optimum pH—The optimum pH for blood or serum diastase will vary slightly depending on the environment in which the diastase functions. In general the zone of optimum pH is in the range of 6.8 to 7.6. In the original Myers-Killian method (1) no buffer was employed, since it was felt that the blood itself should afford a good buffering effect to the digestion mixture. Recently we have reinvestigated this point by comparing the diastatic activity of blood as measured in the Myers-Killian method with the diastatic activity of the same blood when buffered at various pH values from 5.4 to 8.4 with phosphate buffer. The results of such studies show that at the outer limits the amylolytic activity is greatly decreased, whereas in the range around pH 6.8 to 7.6 good digestion occurs. Furthermore the activity of unbuffered whole blood shows as great an amylolytic activity as any of the buffered samples. However, if one uses 1 cc. of serum rather than 2 cc. of whole blood in the digestion mixture, the pH of the mixture increases to above 8.0 and in this instance the unbuffered serum mixture does not give nearly as good an activity as serum buffered at pH 7.2. In their studies on pancreatic amylase Sherman, Caldwell, and Adams (15) found that the optimum pH was close to 7.2, and Free and Myers (8) made the same observation on duodenal contents. 3 cc. of 0.2 M phosphate buffer of pH 7.2 stabilize the pH at 7.2 and the digestion mixture is buffered against either acid or alkali. On the other hand the buffer used by Somogyi (3) contains 3 cc. of 0.1 N HCl and 10 gm. of NaCl in 1 liter and 2 cc. of such a solution are used in his digestion mixture. It would appear that the amount of HCl present in the digestion mixture would have little if any effect in compensating for any loss in CO_2 from the plasma or serum. Experimental measurements with a glass electrode indicated that Somogyi's digestion mixture had approximately the same pH whether or not the HCl-NaCl mixture was present, namely, pH values of 8.0 to 8.3, pH figures which in our hands may reduce the diastatic activity as much as 50 per cent. Furthermore the mere passage of alveolar air (supplying CO_2) through Somogyi's digestion mixture caused changes in pH to values of 7.1 to 7.4, whereas in our phosphate buffer-digestion mixture alveolar air caused no change in pH. It is evident, therefore, that when serum (or plasma) is used it must be buffered to secure a uniform and maximum diastatic activity.

Sodium Chloride Concentration in Digestion Mixture—Below a certain concentration of sodium chloride in the digestion mixture the extent of amylolysis is related to the amount of sodium chloride present. Myers and Free (11) showed for pancreatic amylase in duodenal contents that if the NaCl concentration in the digestion mixture was less than 0.012 M the activity of the amylase was seriously affected. In the Myers and Killian method the amount of whole blood used was 2.0 cc., which can be expected to contribute enough chloride to give an average concentration of 0.018 M. On the other hand in a digestion mixture of 10 cc. in which there is only 1 cc. of serum the molarity of NaCl is around 0.010, which is below the critical level. Therefore it would appear desirable to add sodium chloride to digestion mixtures of 10 cc. volume when only 1 cc. of serum is used, but in mixtures of 10 cc. in which 2 cc. of whole blood were present the addition of NaCl would be unnecessary. We have experimentally established that the addition of NaCl to diastase digestion mixtures such as are employed in the Myers and Killian method does not increase the enzyme activity, whereas addition of NaCl to serum digestion mixtures in which the molarity is only 0.01 M causes a real increase in activity of the enzyme.

Time and Temperature of Incubation—The time of digestion and temperature employed in determinations of blood or serum diastase appear to be quite arbitrary. In the Myers-Killian method a 15 minute digestion period was employed, with 40° as the temperature. It would appear that an interval of 15 minutes for digestion is still quite satisfactory. 40° has been extensively used as the temperature of incubation and since all normal and abnormal values from this laboratory are defined on this basis it seems desirable to continue 40° as the temperature of incubation.

Termination of Enzyme Action and Precipitation of Protein—To determine enzyme activity accurately it is necessary to have some method of effectively stopping the action of the enzyme at the end of a designated interval. Picric acid, which was employed by Myers and Killian (1), is an ideal substance for this purpose, since it requires only a few seconds to saturate a solution with dry picric acid and in such a saturated solution of picric acid, diastatic (or amylolytic) activity is completely inhibited. Furthermore the picric acid effectively precipitates all of the proteins of blood or serum, so that the filtrate can be employed directly for the sugar determination. Such a filtrate is quite stable and may be set aside, if desirable, and the sugar determinations carried out at one's convenience. Somogyi (3) has indicated that his copper-tungstate precipitation of the protein does not fully stop diastase action and that these operations must be carried out without undue delay.

Determination of Reducing Sugar in Diastase Digests—The principal end-product of starch digestion with diastase is maltose or some product that is readily converted to maltose by the common procedures employed in its determination. In the Myers-Killian method, picric acid reduction was employed in the estimation of the amount of maltose formed as a result of amylolysis. It is realized that there are those who object to the use of picric acid as an oxidizing agent in the determination of sugars, but their objections appear to be founded on prejudices rather than facts. It has clearly been shown by a number of investigators, including Pucher and Finch (16) and Myers and Reid (6), that the copper methods such as those of Folin and Wu and Shaffer and Hartmann give only about half as much reduction with maltose as does the picric acid method, whereas the Hagedorn-Jensen ferricyanide method gives only slightly lower figures than the picric acid method. Apparently maltose is fairly completely hydrolyzed with the picric acid and ferricyanide methods but not with the copper methods. Maltose gives approximately 85 to 90 per cent as much reduction of picric acid as does an equal amount of glucose, whereas the copper methods give only approximately 40 to 50 per cent as much reduction with maltose as with glucose. The statement by Somogyi (3) that 40 to 60 per cent reduction expressed as glucose equals 60 to 90 per cent maltose may be true with his copper method but certainly is not true when picric acid is employed to measure the reduction. Somogyi (5) has further indicated his lack of information regarding the nature of the reaction between picric acid and maltose by stating that Myers and Killian have reported results "... which indicated a yield of reducing sugars, computed as maltose, greater than could possibly be obtained if all of the available starch had been split into maltose."

Relation of Enzyme Concentration to Amount of Amylolysis—Myers and Reid (6) have shown that the Myers and Killian method of determining blood diastase gives a proportionality between the amount of enzyme and the amount of reducing sugar formed. Somogyi (3) has not produced any data showing a proportionality between enzyme concentration and amount of reducing sugar formed in his method.

To secure added information on this point determinations of diastatic activity were carried out on the serum of thirty-four normal human subjects with the modified picric acid method described below, except that the original 10 mg. of soluble starch substrate were employed. Simultaneous determinations were also made with the Somogyi method. In each of the methods 1 cc. of serum was employed to suppy the diastase, but despite the fact that the incubation period with the picric acid method was only half as long as with the Somogyi method, the average amount of reducing sugar determined with the picric acid method was 35 per cent greater than that obtained with the copper method (average 1.42 mg. in comparison with 1.05 mg.). If one eliminates one case in which high values were obtained with both methods, the range of values for the two methods calculated in mg. per 1.0 cc. of serum used was 0.70 to 2.17 by the picric acid method and 0.45 to 1.64 by the Somogyi procedure, thus giving means which are the same as the averages given above. It is apparent therefore that 10 mg. of soluble starch are an entirely adequate substrate for 1 cc. of normal human serum.

Fig. 1 shows the relation between concentration of serum diastase and reducing sugar formed in the modified method presented in this paper. It will be seen that up to 15 mg. the amount of reducing sugar is strictly proportional to the enzyme concentration. It is only very rarely that one would encounter activities above this in acute pancreatitis.

Method of Expressing Results—Most enzyme methods record the enzyme activities in some kind of units. Myers and Killian originally took as the diastatic activity the percentage of 10 mg. of soluble starch converted to reducing sugar by 2 cc. of whole blood. Somogyi has taken the mg. of sugar formed by 100 cc. of blood plasma. Observations on the method described in this paper indicate that with 1 cc. of serum the values obtained are essentially the same as those obtained with 2 cc. of whole blood in the Myers-Killian method and for practical purposes may be considered comparable. Since at the present time a variety of methods are employed and each method has its own unit, it is quite difficult to compare results obtained by different investigators. For this reason and in order to facilitate clinical interpretation, Myers and Free (11) have suggested a method whereby the normal average enzyme activity is expressed as 100 per cent. With conditions adjusted to furnish a linear relationship it is very simple to express the enzyme activity in terms of 100 taken as the normal by multiplying the value obtained by a factor. On 57 normal human subjects the average diastase reduction observed for 1 cc. of serum by the method described below was 1.82 mg. By multiplying this value by the factor 55 one obtains 100 per cent as the normal average result. Multiplying any other diastase value, expressed in mg. of reducing sugar per cc. of serum by this same factor 55, will give the value as a percentage of the normal average. Thus if a patient has a serum diastase that produces 3.95 mg. of reducing

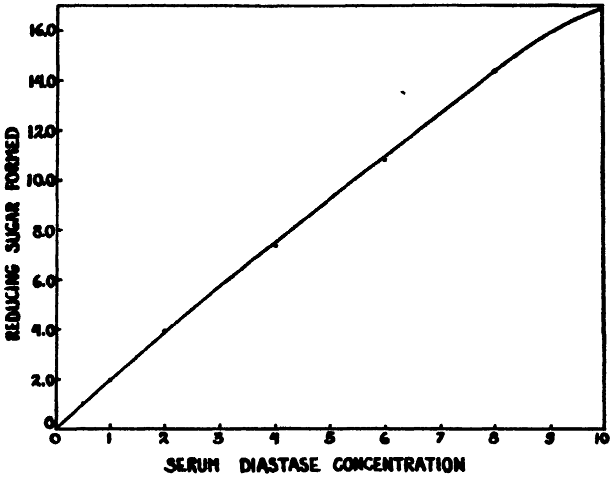

FIG. 1. Relation of enzyme concentration to enzyme activity. Variations in enzyme concentration were obtained by mixing varying ratios of human serum and dog serum. 1 cc. of serum was used in all cases. The abscissa represents the approximate relative amount of enzyme that would be contained in 1, 2, 3, etc., cc. of normal human serum.

sugar per cc. of serum under the conditions of the method, then 3.95 × 55 = 217 per cent, or the level of diastase is 217 per cent of the normal average. We believe that from the standpoint of the clinician this is the simplest way of expressing activities.

Method

Reagents—

1 per cent soluble starch solution, prepared by suspending exactly 1 gm. of soluble starch (Merck, according to Lintner) in 5 cc. of cold water in a 150 cc. beaker and then adding about 90 cc. of boiling water. The starch should dissolve and the solution is then boiled for exactly 1 minute, after which it is made up to 100 cc. in a volumetric flask. This solution should be refrigerated if it is to be used for more than 1 day. With refrigeration the solution can be used about 2 weeks. However, after 2 or 3 days the starch becomes cloudy and in this case the flask containing the starch should be heated for a few seconds in a boiling water bath until the solution becomes clear.

0.2 M phosphate buffer of pH 7.2, prepared by dissolving 7.62 gm. of anhydrous potassium dihydrogen phosphate and 20.45 gm. of disodium hydrogen phosphate in water to give a total volume of 1 liter.

0.5 M sodium chloride solution.

Dry picric acid of reagent quality.

Standard glucose solution, prepared by dissolving 200 mg. of pure glucose in 1 liter of saturated picric acid solution, thus giving 0.6 mg. of glucose per 3 cc. of the standard.

Saturated sodium carbonate solution.

Procedure—5 cc. quantities of soluble starch are pipetted into two 20 × 150 mm. test-tubes and to both tubes are added 3 cc. of 0.2 M phosphate buffer of pH 7.2. 1 cc. of 0.5 M NaCl solution is added to each of the tubes and the tubes then placed in a constant temperature water bath maintained at 40°. One of the tubes serves as a blank (Tube B) and the other tube (A) is the one in which amylolysis is allowed to take place. After the tubes have reached the temperature of the bath, 1 cc. of serum is added to Tube A and digestion allowed to proceed for exactly 15 minutes, after which time about 0.5 gm. of dry solid picric acid is added and the tube thoroughly agitated in order to insure saturation of the solution with picric acid. An equivalent amount of dry picric acid is added to Tube B and after saturation 1 cc. of serum is added, and the tube again agitated. The addition of the serum after saturation with picric acid prevents any amylolysis in the blank tube.

Both tubes are filtered and 3 cc. of filtrate from each are measured into Myers-Bailey sugar tubes along with 1 cc. of saturated sodium carbonate.

3 cc. of the standard glucose solution are measured into a third Myers-Bailey tube along with 1 cc. of saturated sodium carbonate. The tubes are all boiled in a water bath for 20 minutes, after which they are cooled, diluted to 20 cc., and read in a photoelectric colorimeter with a filter having a transmission maximum of 520 mμ. A visual colorimeter can readily be used but in this case it is desirable to dilute the standard to 10 cc. rather than 20 cc. and to dilute the digest and blank so that their color is of the same order of magnitude as that of the standard.

In order to calculate the amount of reducing sugar produced by 1 cc. of serum the following formula can be used,

$$\frac{R}{S} \times \frac{D}{SD} \times 0.6 \times \frac{10}{3} = \text{mg. of reducing sugar formed by 1 cc. serum}$$

In this formula R is the color density of the unknown, S is the color density of the standard, D is the dilution of the unknown, and SD is the dilution of the standard. This calculation is also used for the blank and the value obtained is subtracted from the value obtained for the digest. The relation to the normal activity in terms of 100 may be obtained by multiplying by the factor 55.

SUMMARY

The blood diastase (amylase) method of Myers and Killian, which was designed for whole blood, has been modified for use with serum or plasma and provides optimum sodium chloride, buffer (pH), and substrate concentrations. With this method it is possible to determine accurately serum or plasma diastase over the range found in normals as well as to measure accurately subnormal concentrations and the very high values sometimes encountered in acute pancreatitis.

The advantages of employing picric acid rather than copper as an oxidizing reagent in the practical determination of the sugar formed by amylolysis in blood have again been discussed. With picric acid the reduction in terms of glucose is more than twice as great as with copper for the apparent reason that maltose is hydrolyzed and the figures thus obtained give a better percentage index of the amount of the starch digestion. Furthermore a simple single reagent is employed which serves not only to determine the sugar formed, but also to stop the amylolysis and precipitate the proteins. An added advantage is that the color formed (sodium picramate) is stable and may be read at one's leisure.

From a technical standpoint the method is extremely simple and requires a minimum of reagents, technical skill, and time, but provides an accuracy which exceeds that obtained by most other currently employed methods.

The authors are indebted to Lieutenant Frank J. Grabill, M. C., U. S. N., Lieutenant Donald T. Book, M. C., A. U. S., and Dr. Margaret R. Read for a large amount of experimental work done in connection with this problem.

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THE OPTICAL ACTIVITY OF THE COPPER COMPLEXES OF POLYSACCHARIDES AND SUBSTITUTED METHYL GLUCOSIDES

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(Received for publication, February 29, 1944)

When cellulose, soluble starch, and glycogen are dissolved in cuprammonium hydroxide solution, copper-carbohydrate complexes are formed which exhibit high levo optical rotation. There are, however, glucopyranoside polysaccharides which do not show this behavior. Laminarin, a polysaccharide isolated from the seaweed *Laminaria digitata*, gives a low dextrorotation. The dextran produced by *Leuconostoc dextranicum* shows a dextrorotation in water, but a small levorotation in cuprammonium hydroxide solution. The polysaccharide recently isolated by McIntire, Peterson, and Riker (1) from *Phytomonas tumefaciens* gives a slight levorotation in water, but a high dextrorotation in cuprammonium hydroxide solution.

The first three polysaccharides mentioned are largely composed of pyranoside units linked through the 4 position. Laminarin has been shown by Barry (2) to involve linkage of glycopyranoside units through the 3 position. The *Leuconostoc* dextran has been shown by Peat, Schlüchterer, and Stacey (3) and by Fairhead, Hunter, and Hibbert (4) to be composed of glucopyranose units approximately 90 per cent of which are linked through the 6 position. The linkage in the *Phytomonas* polysaccharide has not yet been established by chemical methods; however, it will be shown that the optical behavior of this polysaccharide in water and cuprammonium solution prompts a prediction that it is composed chiefly of glucose units linked through the 2 position.

Methyl 4-methylglucoside has free hydroxyl groups arranged in the same spatial configuration as cellulose and the 4-linked units of starch and glycogen. It has already been observed (5) that the optical activity of methyl 4-methyl-β-glucoside resembles that of cellulose. It has also been shown that the remarkable levo shift in rotation in cuprammonium solution is not influenced by substitution in the 6 position, but requires that positions 2 and 3 be unsubstituted. It is in agreement with the speculations of earlier workers (6) to conclude that the 4-linked polysaccharides resemble the substituted glucosides by forming copper complexes involving hydroxyl groups in positions 2 and 3 of the glucopyranoside units.

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* This is one of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

The β-methylglucosides of 2-, 3-, and 6-methylglucose were prepared in order to examine their optical behavior in water and cuprammonium hydroxide solutions. These results together with those obtained with the 4-methyl compound are given in Table I. When the optical rotation in water was compared with that in cuprammonium solution, it was found that the latter caused little change in the rotation of the 3-methyl compound, a moderately large dextro shift in the 6-methyl compound, while in the 2-methyl compound a large dextro shift approximately equal in magnitude to the levo shift of the methyl 4-methylglucoside was produced.

In Table II are given the optical rotations of a number of polysaccharides in aqueous and cuprammonium hydroxide solution. The specific rotations are multiplied by the weight of each glucosidic unit to give a value which may be compared with the molecular rotation of the substituted glucosides. Since it is impossible to dissolve native cellulose in water, a degraded cellulose was dissolved in an aqueous solution of trimethylbenzylammonium hydroxide (triton B). Cellulose of high viscosity was employed for the cuprammonium solution. Although starch was reported by Schweizer (7) to be insoluble in cuprammonium solution, a soluble starch preparation dissolved without difficulty.

TABLE I

Optical Rotation of Glucosides in Water and Cuprammonium Hydroxide Solution

Substance	Solvent	Optical rotation	
		$[\alpha]^{25}_{436}$	$[M]^{25}_{436}$
		degrees	*degrees*
Methyl 2-methyl-β-glucoside	Cupra	+985	+204,900
	Water	−69	−14,400
" 3-methyl-β-glucoside	Cupra	−86	−17,900
	Water	−46	−9,600
" 4-methyl-β-glucoside	Cupra	−1008	−209,700
	Water	−36	−7,500
" 6-methyl-β-glucoside	Cupra	+161	+33,500
	Water	−48	−10,000

The optical rotation of a glucoside in water or cuprammonium hydroxide solution is to a considerable extent determined by the α or β configuration around the glucosidic carbon atom. However, in several instances it has been observed that the shift in rotation due to the formation of copper-containing complexes is approximately the same for glucoside pairs differing only in regard to the α or β configuration. In a comparison of the optical behavior of glucosides with that of polysaccharides it seems proper to use the difference or shift in molecular rotation caused by formation of the complex instead of the specific or molecular rotations. In Table III these shifts for the polysaccharides are compared with the shifts for the substituted glucosides. It will be seen that the 4-linked polysaccharides and the 4-substituted glucoside show a large levo shift in rotation, the 3-linked polysaccharide and the 3-substituted glucosides show almost no change in rotation, and the *Phytomonas* polysaccharide resembles the 2-substituted glucoside in showing a large dextro shift in rotation.

TABLE II

Optical Rotation of Polysaccharides in Aqueous and Cuprammonium Hydroxide Solution

Substance	Solvent	Optical rotation	
		$[\alpha]^{25}_{436}$	$[M]^{25}_{436}$
		degrees	*degrees*
Cellulose	Cupra	−1200	−194,400
	Water-triton B (1:1)	−46	−7,500
Starch (soluble)	Cupra	−715	−115,800
	Water	+375	+60,800
Glycogen	Cupra	−597	−96,700
	Water,containing NaCl	+366	+59,300
Laminarin	Cupra	+34	+5,500
	Water	−29	−4,700
Polysaccharide from *Phytomonas tumefaciens*	Cupra	+960	+155,500
	Water	−23	−3,700
Dextran from *Leuconostoc dextranicum*	Cupra	−128	−20,700
	Water	+297	+48,100

The optical behavior of the 6-linked dextran does not closely resemble that of the 6-methylglucosides. Since the polysaccharide is more levorotatory than the glucoside, a part of the difference could be explained by the existence of a small proportion of linkages through the 4 position similar to those found by Levi, Hawkins, and Hibbert (8) in the dextran from the related organism *Leuconostoc mesenteroides.* A second explanation of the difference might be as follows: When the hydroxyl groups on carbon atoms 2, 3, and 4 are free, it is possible that two different complexes of high, but opposite, optical activity may be formed. It has been observed that the complex involving the 2 and 3 hydroxyl groups would be highly levorotatory, while that involving the 3 and 4 positions might be highly dextrorotatory. In solution an equilibrium between the two complexes would be expected and the nature of the groups attached to the glycosidic carbon atom and to carbon atom 5 of the pyranose ring might influence the optical behavior by favoring one complex above the other. Wide differences were actually encountered among the 6-methyl methylglucosides, α- and β-methylglucosides, methyl *D*-xylosides, and levoglucosan, all of which possess the *L*, *D*, *L* configuration for hydroxyl groups 2, 3, and 4, respectively.

It appears that the optical activity of copper-carbohydrate complexes can be used as a rapid means of classifying glucose polysaccharides, and in certain cases valuable information regarding structure can be obtained.

TABLE III

Shift in Molecular Rotation Due to Copper Complex Formation

Substance	$[M]_{436}^{25}$ (cupra)−$[M]_{436}^{25}$ (water)
	degrees
Methyl 2-methyl-β-glucoside	+219,300
Phytomonas polysaccharide	+159,200
Methyl 3-methyl-β-glucoside	−8,300
" 3-methyl-α,β-glucoside	−400
Laminarin	+10,200
Methyl 4-methyl-β-glucoside	−202,200
Cellulose	−186,900*
Starch (soluble)	−176,600
Glycogen	−156,000
Methyl 6-methyl-β-glucoside	+43,500
" 6-methyl-α,β-glucoside	+40,400
" α-glucoside	+24,400
" β-glucoside	+25,000
Levoglucosan < 1,5 > < 1,6 >	−2,800
Methyl α-*D*-xyloside	−25,700
" β-*D*-xyloside	−31,200
Leuconostoc dextranicum dextran	−68,800

* $[M]_{436}^{25}$ (cupra)−$[M]_{436}^{25}$ (water-triton B).

It is possible that upon further refinement the method may become useful as an indication of the purity of polysaccharides, or as a test for branching, or for more than one type of linkage.

EXPERIMENTAL

The magnitude of the rotation of complex-forming substances is dependent upon the relationship between concentration of copper and carbohydrate. To keep this ratio constant all observations were made on approximately 0.03 M glucoside solutions or on 0.5 per cent polysaccharide solutions in cuprammonium hydroxide solution containing approximately

0.24 mole of copper per liter. Aqueous solutions of similar concentrations were employed for comparison.

The cuprammonium hydroxide solutions contained 15 gm. of copper, 240 gm. of ammonia, and 1 gm. of sucrose (9) or glycerol per liter. The sucrose-containing solution had an optical rotation of +0.09° (Hg blue line, 0.5 dm.) which was subtracted from all readings when this solution was used.

The mercury blue line (436 mμ) was isolated for aqueous solutions by Corning Filters 511 and 038. For cuprammonium solutions only Filter 038 is required, since the longer wave-lengths are completely absorbed by the solution. The source of light was a General Electric high pressure mercury arc. All observations were made in a Gaertner polarimeter with 0.5 dm. tubes for cuprammonium solutions and 1 or 2 dm. tubes for aqueous solutions.

Methyl 2-methyl-β-glucoside was prepared from crystalline 2-methylglucose by the procedure of Oldham (10). M.p. 94–95° (corrected); $[\alpha]_D^{25} = -37°$ (H_2O).

Methyl 3-methyl-β-glucoside was prepared from crystalline 3-methylglucose by the procedure of Oldham (10). The melting point of the tribenzoate was 132–134° (corrected), a value higher than the 125–126° reported. The rotation of the tribenzoate was $[\alpha]_D^{25} = +16°$ ($CHCl_3$), and of the free glucoside $[\alpha]_D^{25} = -27°$ (H_2O). A mixture of methyl 3-methyl-α- and β-glucosides, $[\alpha]_{436}^{25} = +223°$ (H_2O, $c = 0.6$), +221° (cupra, $c = 0.6$), was prepared by deacetylation of the triacetate. The triacetate was prepared by boiling 3-methyldiacetoneglucose for 24 hours in methanol containing 3.2 per cent hydrogen chloride, evaporating to dryness *in vacuo*, and acetylating the product. The acetate was purified by distillation at 190–195° at 2 mm. pressure.

Methyl 4-methyl-β-glucoside triacetate, m.p. 107–108° (corrected), from the collection of the late Dr. P. A. Levene was dissolved and deacetylated simultaneously in the cuprammonium solution. The aqueous solution was prepared by dissolving the crystals in a slight excess of warm N sodium hydroxide and neutralizing with dilute acetic acid after saponification was judged to be complete. The aqueous solution contained approximately 1 per cent sodium acetate.

Methyl 6-methyl-β-glucoside solutions were prepared in the manner described above from the crystalline triacetate which was prepared according to the directions of Helferich and Gunther (11). The free glucoside gave an optical rotation of $[\alpha]_D^{25} = -27°$ (H_2O, containing 1 per cent NaOAc). Methyl 6-methyl-α- and β-glucosides were also prepared by refluxing 500 mg. of crystalline 6-methylglucose in 20 ml. of methanol containing 1 per cent dry hydrogen chloride for 2.5 hours. After removal of acid with ethereal diazomethane the solution was evaporated to dryness and the residue distilled in a micro sublimation outfit at 150° and 0.05 mm. $[\alpha]^{25}_{436} = +132°$ (H_2O); $+326°$ (cupra).

Analysis—$C_8H_{16}O_6$ (208). Calculated, OCH_3 29.8; found, 29.5, 29.3

Samples of methyl α- and β-*D*-xyloside, methyl α- and β-glucoside, and levoglucosan having melting points in agreement with values given in the literature were employed. The optical rotations of these substances are given in Table IV.

A sample of polysaccharide from *Phytomonas tumefaciens* was kindly supplied by Professor W. H. Peterson. This material has been shown to be composed of glucopyranose units (1). The sample for rotation was dried in a vacuum at 100°. $[\alpha]^{25}_{D} = -11°$ (H_2O).

TABLE IV

Optical Rotation of Certain Substances with D Line and Hg Blue Line

Substance	Solvent	Specific rotation (25°)	
		D line (589 mμ)	Hg blue line (436 mμ)
		degrees	*degrees*
Methyl α-*D*-xyloside	Water	+155	+295
	Cupra		+138
" β-*D*-xyloside	Water	−60	−127
	Cupra		−317
" α-glucoside	Water		+306
	Cupra		+432
" β-glucoside	Water		−62
	Cupra		+67
Levoglucosan	Water	−65	−127
	Cupra		−144

Laminarin was prepared from *Laminaria digitata* collected in the early summer of 1939 at the Marine Biological Laboratory, Woods Hole, Massachusetts. The method of isolation has been described by Barry (12). The rotations were calculated upon a moisture-free basis. $[\alpha]^{25}_{D} = -14°$ (H_2O).

Cellulose in the form of cotton fiber was purified by extraction with alcohol and 1 per cent sodium hydroxide solution. This material was used for the cuprammonium solution. Before being dissolved in triton B the purified cellulose was partially hydrolyzed by heating for 1 hour in a water bath at 100° with N hydrochloric acid, filtered, washed, and dried. The triton B solution of cellulose was diluted with an equal volume of water, and centrifuged to remove a slight turbidity. Rotations were calculated upon a moisture-free basis. $[\alpha]^{25}_{D} = -16°$ (water-triton B, 1:1).

Soluble starch was prepared according to the directions of Morrow and Sandstrom (13). The cuprammonium solution was centrifuged to remove a slight cloudiness. The rotations were calculated upon a moisture-free basis.

Glycogen was dried to constant weight at 100° in a vacuum. The aqueous solution was prepared by dissolving the glycogen in N sodium hydroxide and adding an equivalent quantity of hydrochloric acid. $[\alpha]_D^{25} = +184°$ (H_2O containing 3 per cent NaCl).

A sample of the dextran synthesized by the action of *Leuconostoc dextranicum* upon sucrose solution was kindly supplied by Professor Harold Hibbert. The material formed a gel which dissolved slowly in cuprammonium. The solutions were centrifuged to remove a trace of insoluble material. The rotations were calculated upon a moisture-free basis. $[\alpha]_D^{25} = +151°$ (H_2O).

SUMMARY

It has been found that the four possible monomethyl β-methylglucopyranosides show widely different optical behavior when dissolved in cuprammonium hydroxide solution.

The optical activity of methyl 2-methyl-β-glucoside in water and cuprammonium so closely resembles that of the polysaccharide from *Phytomonas tumefaciens* that it is suggested that this polysaccharide is composed of glucopyranose units linked chiefly through the 2 position. The optical behavior of a 3-linked polysaccharide and several 4-linked polysaccharides is similar to that of the correspondingly substituted methyl glucosides.

The shift in the optical rotation of glucopyranoside polysaccharides upon being dissolved in cuprammonium hydroxide solution may be used to classify glucose polysaccharides and may in certain instances furnish information regarding the structure of the polysaccharide.

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THE TRYPTOPHANE AND TYROSINE CONTENT OF PEANUT PROTEINS*

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(Received for publication, February 25, 1944)

The feeding experiments of Baernstein (1) with peanut proteins indicated that conarachin was a complete protein for growth in young rats, but that arachin was deficient in tryptophane as well as in methionine. In view of the lack of agreement on the tryptophane content of the peanut proteins, as shown by the results of Jones, Gersdorff, and Moeller (2), and those of Milone and Everitt (3), who used modifications of the May and Rose method (4), on the one hand, and those of Kotasthane and Narayana (5), who used the Folin and Marenzi method (6), on the other, it was felt that this discrepancy should be clarified. Looney (7) expressed lack of confidence in the results obtained by most of the methods for tryptophane, especially when the May and Rose method was used. Shaw and McFarlane (8) showed that by the May and Rose method erroneous results were obtained and confirmed the reliability of their modification of the glyoxylic acid method (9).

EXPERIMENTAL

The arachin and conarachin used were the preparations referred to in an earlier paper (10). Jones and Horn (11) found 40.5 per cent crude protein (N × 5.5) in an oil-free peanut meal. Repeated extraction of the meal with 10 per cent sodium chloride solution gave an extract containing 87 per cent of the total crude protein. Examination of this extract indicated the presence of arachin and conarachin to the extent of 61.2 and 21.5 per cent, respectively, of the total crude protein. This is equivalent to 25 and 8.7 per cent of arachin and conarachin, respectively, based on the weight of the oil-free peanut meal.

In a report from this Station (12) it has been shown that the protein of oil-free unheated peanut meal can be dispersed in water up to the extent of from 80 to 93 per cent of the total crude protein. Of this protein dispersed in water, from 80 to 88 per cent may be precipitated by saturating the solution with carbon dioxide. This easily prepared protein preparation, which should be comparable to peanut total globulin in composition, is convenient for use as representative of the total peanut protein.

Sound, clean, shelled peanuts of Strain 177-23 (Philippine White × North

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* Published with the approval of the Director as paper No. 122, Journal Series.

Carolina Runner) were ground and extracted with petroleum ether. Practically all of the seed-coat was removed by screening. The oil-free meal contained 43.5 per cent crude protein. The water-dispersible protein was prepared by stirring 1 part of this meal with 30 parts of distilled water for an hour. Then the supernatant solution was centrifuged and filtered. A few ml. of ether and toluene were added to prevent foaming and bacterial decomposition. After the protein solution was saturated with carbon dioxide, it was stored in a closed container overnight. The supernatant liquid was decanted and rejected. The remaining protein was separated by centrifuging, was then mixed with water saturated with carbon dioxide, and stored at 5° overnight. After the protein was separated again with the centrifuge, it was dried with alcohol and ether in the usual manner. Water-dispersible peanut protein prepared in this way was found to contain 0.33 per cent ash on a moisture-free basis, and 17.46 per cent of nitrogen on an ash- and moisture-free basis. Examination of this protein by the method of Palmer, Smyth, and Meyer (13) indicated that it contained 0.16 per cent hexosamine.

Glyoxylic Acid Method—The earliest estimations of tryptophane by the glyoxylic acid method in this laboratory were not entirely satisfactory. Shaw and McFarlane observed (9) that when certain samples of concentrated sulfuric acid were employed a yellowish brown color developed in the blank determination. Several reagent grades of sulfuric acid were tried, but none was entirely satisfactory. Distilling the acid in the first apparatus that was used was not satisfactory.

Preliminary experiments indicated that very small amounts of nitrates or nitrites might be responsible for the difficulty. It was found that in the presence of free tryptophane in the blank determination a yellow-brown color was produced by traces of either nitrates or nitrites. Moreover, a similar yellow-brown color was not produced when alkaline solutions of unhydrolyzed proteins were used in place of the standard tryptophane solution. Since the calibration curves are prepared with standard solutions of free tryptophane, this introduces a serious source of error. In addition, it was found that very small amounts of either nitrates or nitrites lowered the amount of blue color developed with both the free tryptophane and the solutions of unhydrolyzed protein.

The influence of nitrates, nitrites, and ammonia on the recovery of tryptophane in the glyoxylic acid method is illustrated in Table I. The sulfuric acid used was a very pure sample of redistilled acid obtained from the J. T. Baker Chemical Company.[1] Eaton[2] found "in distilling ordinary C.P. acid containing a trace of nitrate, nitric acid could not be detected with diphenylamine until more than half of the acid had been distilled."

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[1] Courtesy of G. B. Hafer and F. C. Eaton.

[2] Eaton, F. C., personal communication.

For redistillation a 6 liter Yoe conductivity water still which had a double vapor trap was used. The upper part of the flask, the side tube, and vapor trap were covered with asbestos paper to minimize radiation. To 4.5 liters of acid for Kjeldahl analysis in the distilling flask were added, to prevent bumping, a few pieces of alundum which had been treated with hot sulfuric acid, rinsed in distilled water, and then calcined. The first 125 ml. of distillate were rejected. Three fractions of 1780, 660, and 280 ml., respectively, were collected. All three of the fractions gave good blanks and were entirely satisfactory.

In Table II are shown the results obtained on peanut proteins for tryptophane by the glyoxylic acid method (9), and for tyrosine by the method of Folin and Marenzi (6) as outlined by Block and Bolling (14), but adapted for use with the spectrophotometer. The transmission was determined at λ 540 and 520 mμ for tryptophane and at λ 480 mμ for tyrosine with a Coleman No. 11 spectrophotometer. Included for comparison are the results found by several other investigators.

TABLE I

Influence of Nitrates, Nitrites, and Ammonia on Recovery of Tryptophane

The amount of nitrate, nitrite, or ammonia ion was added as a standard solution of sodium nitrate, sodium nitrite, or ammonium sulfate before the volume was adjusted to 2 ml. and the copper sulfate, glyoxylic acid, and 5 ml. of sulfuric acid added.

Weight of nitrate, nitrite, or ammonia added	Tryptophane recovered after addition of		
	Nitrate	Nitrite	Ammonia
mg.	*per cent*	*per cent*	*per cent*
0	100	100	100
0.002	98.5	103.6	
0.005	98.5	87.4	
0.01	73.1	44.3	
0.02	26.2	5.5	98.8
0.04		0	
0.05			101.2
0.10			99.4
0.15			98.8
0.20			98.8

Eckert Method—In order to check further on the tryptophane content of the peanut proteins, the method of Eckert (15) was used. The proteins were hydrolyzed with 5 N sodium hydroxide under a reflux at 125°. At the end of the period of hydrolysis, and while the solution was hot, enough 12 N hydrochloric acid was added to neutralize the alkali and make the solution 1.2 N in hydrochloric acid, when diluted to the required final volume. The hydrolysate was treated with 400 mg. of kaolin per 100 ml. of hydrolysate, centrifuged, and filtered through dry filter paper. From this point on the method was the same as that of Eckert, except that it was found to be essential to determine the factor for the color produced by tryptophane each

TABLE II

Comparison of Tryptophane Results by Several Methods, and Tyrosine Content of Proteins

Corrected for moisture and ash.

Protein	Amino acid	Results of				
		Author	Kotasthane and Narayana†	Jones, Gersdorf, and Moeller‡	Milone and Everitt‡	Holiday
		*per cent**	*per cent*	*per cent*	*per cent*	*per cent*
Arachin	Tryptophane	0.68	0.66–0.69	0.88	1.80	
	Tyrosine	5.68	5.43–5.69	5.50		
Conarachin	Tryptophane	0.91	0.93–1.07	2.13	1.78	
	Tyrosine	2.86	3.09–3.23			
Total globulin	Tryptophane		0.58–0.66	1.00		
	Tyrosine		4.80–5.48			
Water-dispersible peanut protein	Tryptophane	0.79				
	Tyrosine	4.80				
Casein	Tryptophane	1.03§		2.2	2.44	0.85–1.26
	Tyrosine	5.70§				6.45–7.5

* Averages of several determinations.
† Prepared from two varieties, local and Spanish.
‡ Prepared from Virginia peanuts.
§ Labco vitamin-free casein.

TABLE III

Tryptophane by Eckert Method

Corrected for moisture and ash.

Protein	Length of hydrolysis			
	6 hrs	7 hrs.	24 hrs	48 hrs.
	per cent	*per cent*	*per cent*	*per cent*
Arachin	0.66		0.74	0.70
Conarachin	0.72	0.76	0.70	0.71
Water-dispersible peanut protein	0.77		0.82	0.82
Casein	1.02			

time determinations of tryptophane in proteins were made. The transmission at λ 560 mμ was determined with the spectrophotometer. The results for the tryptophane content of the proteins hydrolyzed for different lengths of time are shown in Table III.

DISCUSSION

It is evident that nitrates and nitrites must be excluded from both the reagents and the proteins when the glyoxylic acid method is used for the determination of tryptophane. If this precaution is exercised, the glyoxylic acid method appears to be as reliable as it is convenient.

The Eckert method for tryptophane confirms the tryptophane results found by the glyoxylic acid method in all cases except with conarachin. The results for conarachin obtained by the Eckert method are obviously erroneous, although it is not possible to give an explanation at this time. With this method the results found were not always precise.

The tryptophane results found for casein by both the glyoxylic acid method and the Eckert method agree with that found by Holiday (16) by ultraviolet spectrophotometry. It appears that the *p*-dimethylbenzaldehyde method for the estimation of tryptophane gives erroneous results.

It is probable that there is little difference in the composition of the proteins prepared from different strains of peanuts. The peanut protein prepared by dispersion in water may prove to be comparable to total globulin in composition.

SUMMARY

Nitrates and nitrites, if present in sulfuric acid to the extent of 0.0001 per cent, interfere with the determination of tryptophane by the glyoxylic acid method. A satisfactory grade of sulfuric acid can be obtained by distilling the acid in a conductivity water still.

The results are given for the tryptophane and tyrosine content of the peanut proteins.

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INTERFERENCE IN THE DETERMINATION OF THIAMINE WITH THE DIAZOTIZED *p*-AMINOACETOPHENONE REAGENT*

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(Received for publication, March 11, 1944)

In an analysis of the effect of common enzyme inhibitors on the destruction of thiamine by the Chastek paralysis factor of fish tissues (1) an interference of the inhibitor compounds with the method for thiamine determination was encountered. In these experiments control solutions containing thiamine and the inhibitor yielded less color with the Melnick and Field (2) colorimetric procedure than did solutions containing only thiamine.

Interference with the activity of the diazotized *p*-aminoacetophenone reagent has been previously reported. For example, Prebluda and McCollum (3) in the original description of the reagent stated that, "High salt concentrations and metallic ions were found to influence . . . the reagent." However, the influence of high salt concentrations (sodium sulfate, sodium chloride, and potassium chloride) was overcome by Melnick and Field (2) with the inclusion of alcohol and phenol in the reaction mixture. Of equal interest is the interference resulting when ascorbic acid is present in relatively large amounts (4, 5).

The inhibitor compounds employed in characterizing enzymes and enzyme reactions represent a wide variety of inorganic and organic substances, including heavy metal salts, oxidizing and reducing agents, organic compounds of highly specific reactivity, as well as agents with less well understood actions. In view of the possibility that interference from these or similar compounds may be of importance in other instances in which the colorimetric method is used, the interference encountered and a more detailed analysis of certain phases are presented at this time.

EXPERIMENTAL

Thiamine determinations were made by the method described by Melnick and Field (2) with the minor variations indicated. In the majority of the experiments, 5 ml. of 5×10^{-4} M thiamine in 0.04 M phosphate buffer, pH 7.4, with or without the inhibitor compound to which had been added

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* The authors are indebted to the Rochester Brewing Company, Inc., for making available a research fund in support of this investigation.

5 ml. of 10 per cent trichloroacetic acid, served as the solutions for analysis. The phosphate and trichloroacetic acid were present, since the experimental solutions were originally derived from work with the fish principle. However, numerous experiments demonstrated the presence of these substances to be without influence on the interference measured.

From the total volume of 10 ml., 2 ml. were transferred to small mouth 50 ml. centrifuge tubes containing 3 ml. of water and 5 ml. of the Melnick-Field alcohol-phenol mixture. 10 ml. of the diazotized *p*-aminoacetophenone reagent were added, the colored complex subsequently being extracted with 10 ml. of xylene and the color read in the Klett-Summerson photoelectric colorimeter with the No. 52 filter. For standards of comparison, thiamine solutions of the same composition were used, except that the interfering chemicals were omitted.

TABLE I

Interference with Diazotized Aminoacetophenone Reaction for Thiamine

Compound	Concentration	Thiamine value
	moles per l. × *10^3*	*per cent of standard*
Mercuric chloride	1.0	0
Silver nitrate	10.0	0
Sodium tungstate	10.0	0
Potassium ferricyanide	10.0	0
Hydroxylamine	10.0	0
Hydrogen sulfide	Excess	4.0
Cysteine	10.0	26.2
Iodine	10.0	52.0
Sodium oxalate	1.0	91.2
" fluoride	1.0	92.1
Iodoacetic acid	1.0	92.1
Sodium sulfite	20.0	94.3
Potassium cyanide	1.0	94.3
Sodium pyrophosphate	10.0	96.3
Ferric chloride	1.0	96.5
Zinc chloride	1.0	97.8
Acetone	Excess	98.8
Arsenious acid	10.0	100
Malonic acid	10.0	100
Cupric sulfate	1.0	101
Thiamine concentration, 5.0×10^{-4} M		

The results obtained with the compounds examined are illustrated in Table I, in which are represented the highest concentrations used and the resultant comparison with the thiamine standard. It is evident that the heavy metals, ferricyanide, hydroxylamine, hydrogen sulfide, and cysteine produce the greatest effect. Iodine is somewhat less effective and the other compounds produce only very little or no effect in the concentrations used.

That the interference encountered is a function of the relative concentration of the interfering compound and thiamine is illustrated in the case of mercuric chloride in Fig. 1, in which the amount of color derived from thiamine is plotted against concentration of the mercury compound. The same relationship has been observed in the case of each of the substances influencing the reaction, as is further illustrated in the values obtained with cysteine (Table II, second column).

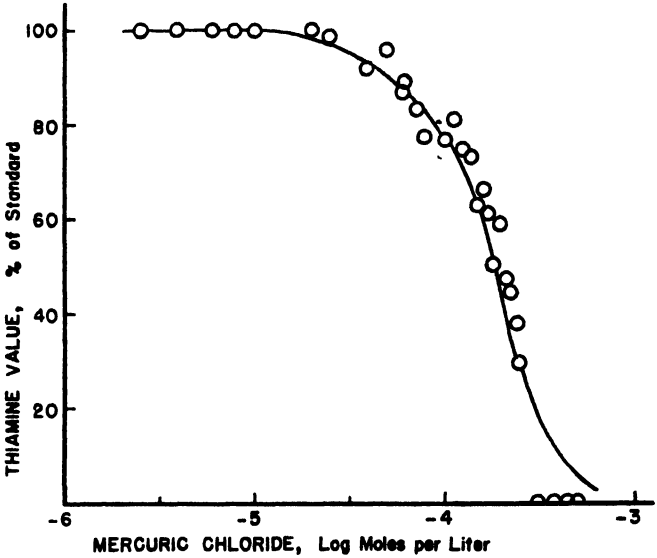

FIG. 1. The effect of increasing mercuric chloride concentrations on the thiamine value. 5 ml. of 5 × 10^{-4} M thiamine containing the proper amount of mercuric chloride were used. Each value is the average of two to five determinations.

The mechanism of the resultant production of decreased color is not entirely clear in each case, although suggestions may be made in certain instances. For example, the reduced color value obtained with 0.02 M sodium sulfite may probably be explained on the basis of the well known destruction of thiamine by sulfite. Concentrations of this reagent which permitted complete recovery of thiamine under the usual conditions yielded significantly lower values when the mixtures were incubated for 2 hours at 37.5°, as would be expected if the above explanation is the correct one. The trichloroacetic acid was added immediately after the incubation and the determinations were made without delay. Table II shows that not only did the increase in temperature decrease the thiamine recovery, but also that the decrease is proportional to the sulfite concentration. On the other hand, in the case of cysteine the incubation treatment did not materially alter the values obtained at room temperature.

The interference obtained with mercuric chloride and with iodine may be assumed to be of a different type, for each of these reagents is known to precipitate thiamine from solution. Although no visible precipitation occurred, it is likely that the complexes were formed and that they are of such a nature as to prevent coupling with the diazotized *p*-aminoacetophenone. Support for this view-point is available in the finding that the addition of mercuric chloride after the addition of the diazotized reagent is without significant influence on the color obtained. The same amount of mercuric chloride added just prior to the addition of the reagent produced the usual interference, as may be seen by comparison of the values in Table III.

TABLE II

Sodium Sulfite and Cysteine Interference

Concentration	Thiamine value*	
	Unincubated	Incubated
	Sodium sulfite	
moles per l. × 10^3	*per cent of standard*	*per cent of standard*
20.0	94.3	74.7
10.0	102	87.1
5.0	101	89.7
2.0	100	92.0
1.0	101	93.2
0.5	103	96.6
0.2	99	96.0
	Cysteine	
10.0	26.2	28.4
1.0	83.8	89.4
0.1	100	93.8
0.01	100	95.4

* Unincubated, 2 hours at room temperature; incubated, 2 hours at 37.5°, immediately prior to the addition of trichloroacetic acid and analysis.

Since the thiazole portion of the thiamine molecule is responsible for the formation of the colored complex with the Prebluda-McCollum reagent, the effect of mercuric chloride on the color obtained with 4-methyl-5-β-hydroxyethylthiazole methiodide[1] was determined. It is evident from the values in Table IV that interference likewise occurs.

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[1] The authors gratefully acknowledge a generous supply of this compound and of thiamine from the Medical Department of Merck and Company, Inc., for use in these experiments.

While it may be argued that the compounds yielding a positive effect are seldom encountered in thiamine-containing materials, the possibility of similar difficulties being present should be borne in mind. This point may be illustrated by the results of an assay of the thiamine content of one lot of commercial tablets containing the synthetic water-soluble vitamins. The dissolved tablets when assayed by either the Melnick and Field procedure or the fermentation method of Schultz *et al.* (6) yielded values of 0 to 0.25 mg. of thiamine instead of the 1.0 mg. expected. The preliminary removal of the coating made no difference in the values obtained. The interior consisted of approximately 190 mg. of cocoa as filler and 30 mg. of ascorbic acid in addition to various amounts of the other synthetic B vitamins. Consequently separate 1 mg. portions of thiamine were mixed with 190 mg. of cocoa and 30 mg. of ascorbic acid from the same materials used in compounding the tablets. The fermentation method showed the presence of 0.56 mg. of thiamine in the former mixture and 0.46 mg. in the latter, similar values being obtained with the Melnick-Field method.

TABLE III

Addition of $HgCl_2$ before and after Diazonium Reagent

The 0.2 ml. of mercury compound was added to the thiamine aliquot in the water-alcohol-phenol mixture contained in the centrifuge tubes either just before or just after the addition of the diazotized reagent. Thorough mixing between additions was accomplished.

$HgCl_2$ added, 0.2 ml.	Thiamine value	
	Before	After
× 10^3 M	*per cent of standard*	*per cent of standard*
1.25	0	96.3
0.5	67.7	96.3
0.05	100	100

TABLE IV

Interference with Thiazolium Methiodide Color

The 2 ml. aliquots analyzed contained 2.5 × 10^{-4} M 4-methyl-5-β-hydroxyethylthiazole methiodide and were compared with a standard of the same compound.

$HgCl_2$ concentration	Thiazole value
mole per l. × 10^3	*per cent of standard*
0.5	0
0.25	1.6
0.1	60.9
0.01	100

These results not only confirm the influence of ascorbic acid previously referred to, but also indicate the deleterious effect of cocoa on the thiamine molecule. The practical implications are, of course, obvious.

The results of these experiments demonstrate difficulties which may occur in making thiamine assays. It is to be expected that certain ones may be circumvented by the proper combination of procedures, although investigations to date indicate that a single uniform procedure is not entirely applicable in all cases.

The interference observed raises questions of both theoretical and practical interest. Although the undesirable influence of ascorbic acid in modifications of the Melnick and Field method has been successfully overcome (4, 5), the results of these experiments suggest that the mixing of the two vitamins under widely varying conditions may necessitate analytical demonstration of the expected thiamine potency. In view of the decreased values obtained in the presence of cysteine an investigation of the effect of the glutathione in yeast or tissues on the thiamine content should prove of interest. These and additional points justify further search for and study of interfering substances not only for the purpose of clarifying the assay procedures but also for further elucidation of the chemical behavior of the thiamine molecule.

SUMMARY

1. The presence of heavy metal salts, potassium ferricyanide, hydroxylamine, hydrogen sulfide, cysteine, iodine, or sodium sulfite markedly influences the reactivity of thiamine with the diazotized *p*-aminoacetophenone reagent. Consequently, lower values are obtained in the colorimetric determination of thiamine.

2. That the extent of the phenomenon is a function of the concentration of extraneous compound has been demonstrated, evidence in the case of mercuric chloride, sodium sulfite, and cysteine being presented.

3. Although adequate explanation of the observed results is available in some cases, in others a satisfactory mechanism awaits further investigation.

4. The possibility of theoretical and practical implications of relationships of this type is illustrated with the results of thiamine assays which show the deleterious effect of cocoa on the thiamine values obtained by both the Melnick and Field and fermentation methods. Evidence of the interference of ascorbic acid in the fermentation method is also presented.

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INTERRELATIONSHIP BETWEEN THIAMINE AND RIBOFLAVIN IN THE LIVER[*]

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(Received for publication, March 22, 1944)

The possibility of an interrelationship between vitamins of the B group was first suggested by clinical observations. Scandinavian authors (1–3) have reported that treatment of multiple deficiencies with thiamine alone precipitated the appearance of lesions characteristic of nicotinic acid deficiency, and Sydenstricker (4) encountered the enhancement of certain signs of deficiency in pellagrins following treatment with nicotinic acid. These and other observations, taken as evidence of an interdependence between individual vitamins of the B group, stimulated speculation as to whether the lack of one vitamin or an overdosage of an individual member of the vitamin B complex might derange the requirements for others ("vitamin balance"). Experimental data on an interdependence among the B vitamins are meager. Unna and Clark (5) were unable to show that excessive amounts of individual vitamins given to rats deficient in one or more factors of the vitamin B complex aggravate the manifestations of the specific deficiency. Klopp, Abels, and Rhoads (6) found a transitory increase in riboflavin excretion in men treated with large doses of thiamine. Prolonged administration of thiamine, however, failed to produce any clinical evidence of riboflavin deficiency.

Sure and Ford (7) reported an increase in riboflavin excretion in the urine of thiamine-deficient rats. They suggested that thiamine deficiency, by affecting the utilization of riboflavin, may produce riboflavin deficiency in man. Ferrebee and Weissman (8), however, in studies on thiamine-deficient patients concluded that thiamine deficiency is not of clinical significance in the production of riboflavin deficiency. In thiamine-deficient rats, they observed changes in riboflavin metabolism only in the terminal stages of the deficiency, which were regarded as non-specific.

Supplee, Jensen, Bender, and Kahlenberg (9) made the observation that the concentration of riboflavin in the liver increased temporarily following ingesting of food. This increase was not observed in thiamine-deficient and in pantothenic acid-deficient rats. Since the administration of these two vitamins promptly restored this transient hepatic increase in riboflavin during digestion, it appears that both thiamine and pantothenic acid exert an influence upon the mobilization of riboflavin.

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[*] This work was carried out with the aid of a grant from the Nutrition Foundation, Inc.

In the course of a study of the effect of vitamin deficiencies on the metabolism of estradiol (10), data on the thiamine and riboflavin content of the liver of these animals were obtained which suggested an interrelationship between these two vitamins. Further studies of such an interrelation between thiamine and riboflavin were undertaken, and the results are reported in this communication.

Methods

The composition of the various diets employed in this study is given in Table I. Animals maintained on Diet 18 were given by stomach tube a daily supplement of B vitamins as indicated in Table I. Diet 18 devoid of B vitamins served in the production of deficiencies in the entire B complex as well as in individual factors such as thiamine, riboflavin, pyridoxine, or pantothenic acid. This was accomplished by omitting either all vitamins or the specific vitamin in question from the daily supplement. In Diet 18A the B vitamins were admixed in the diet, and thiamine or riboflavin deficiency was obtained by omitting thiamine or riboflavin respectively from the diet.

Thiamine deficiency was also produced in rats maintained on Diet 20 in which autoclaved yeast was used to supply B vitamins other than thiamine. The small amount of non-autoclaved brewers' yeast is responsible for a thiamine content of 0.2 γ per gm. of diet as measured by the thiochrome method. Control animals received a daily supplement of 40 γ of thiamine. Through the courtesy of Dr. D. W. Woolley, a group of thiamine-deficient mice was obtained in which the deficiency was produced by the feeding of pyrithiamine (11).

Diet 23 was used for the production of biotin deficiency. Control animals received 2 γ of biotin daily. Vitamin A deficiencies were obtained on Diet 44. Animals maintained on Diet 18 served as controls for the vitamin A-deficient rats.

Male and female rats of two different strains (albino and piebald, Sprague-Dawley) raised in the laboratory were used. At 21 days of age the animals were placed in individual cages and given one of the experimental diets. Food and water were consumed *ad libitum*. The animals were continued on the deficient diets until severe signs of deficiency developed. Every experiment was controlled by a group of litter mates on the same ration supplemented with an adequate amount of the particular vitamin. Furthermore, with thiamine- and riboflavin-deficient animals paired feeding experiments were carried out in which the control animals were restricted to the food intake of deficient animals. Deficient and control animals were sacrificed simultaneously. The weight of the liver and the kidneys was recorded and the moisture content of the liver was determined.

The thiamine and riboflavin content of the liver was determined in individual assays by the thiochrome method of Hennessy and Cerecedo (12) and by a modification of the fluorometric method of Hodson and Norris (13) respectively.

TABLE I

Composition of Experimental Diets

Components	Diet 18	Diet 44	Diet 18A	Diet 20	Diet 23
	Gm. per 100 gm.				
Casein, vitamin-free	18	18	18		15
" technical				16	
Dried egg albumin					15
Dextrose	68	70	68		
Sucrose					52
Corn-starch				60	
Vegetable fat (Crisco)	8	8	8	9	10
Salts U. S. P. XI No. 1	4	4	4	4	4
Cod liver oil	2		2	2	2
Autoclaved yeast				9	
Non-autoclaved brewers' yeast				0.2	
Dried beef liver					2
	Fed separately, mg. per rat daily		Mg. per 100 gm.		
Thiamine	0.04	0.04	1		0.8
Riboflavin	0.08	0.08	2		1.6
Nicotinamide	0.5	0.5	10		10
Pyridoxine	0.04	0.04	1		0.8
Ca pantothenate	0.2	0.2	10		5
Choline chloride	5	5	100		100
	Weekly supplements per animal				
α-Tocopherol, *mg.*	5	5	5		
Vitamin D concentrate, *i.u.*		2000			

Results

Simultaneous determinations of the thiamine and riboflavin concentration in the liver were carried out on a total of more than 200 animals. The results are grouped according to the various deficiencies in Tables II to V.

No significant difference in the vitamin content of the liver was found between the sexes nor between the two strains of rats. Also, the concentrations of thiamine and riboflavin in the liver were not influenced by the age of the animals. They remained practically unchanged throughout a period of 3 months after weaning with the exception that occasionally during the first 2 weeks slightly lower values were found. The concentration of riboflavin, however, was dependent on the type of diet used. A greater concentration of this vitamin was found in the livers of animals maintained on Diets 23 and 20 than in those on Diets 18 and 18A (Tables II and III). Since Diets 23 and 20, owing to the addition of egg white or autoclaved yeast, contain more protein than Diet 18, it appears possible that the riboflavin concentration of the liver may be dependent upon the dietary protein. In general, the riboflavin values were quite uniform in any given group and varied little from one experiment to another. On the other hand, the thiamine values were more erratic.

TABLE II

Effect of Deficiencies in Biotin, Pyridoxine, Pantothenic Acid, and Vitamin A upon Concentration of Riboflavin and Thiamine in Liver

Deficiency	Diet No.	No. of animals	Days on diet	Body weight		Riboflavin		Thiamine	
				Average	Range	Average	Range	Average	Range
				gm.	*gm.*	*γ per gm*	*γ per gm.*	*γ per gm*	*γ per gm.*
Biotin	23	6	35–48	135	100–154	28.4	25.8–33.5		
Control (2 γ daily)	23	4	35–48	253	195–284	30.0	27.6–33.4		
Pyridoxine. .	18	15	49–60	60	46– 96	22.5	18.3–25.8	4.9	2.8–6.8
Control (40 γ daily)	18	9	49–60	170	144–207	23.4	19.6–26.8	4.1	2.8–6.8
Pantothenic acid..	18	7	52–60	83	61–107	24.6	19.8–30.0	5.8	4.5–9.3
Control (200 γ daily).........	18	7	52–60	162	144–196	23.5	19.6–28.8	5.6	3.3–6.8
Vitamin A.......	44	10	48–60	104	91–138	23.3	17.1–26.4		
Control (2% cod liver oil)..	18	5	48–60	210	151–254	24.3	20.7–29		

The nutritional state *per se* had little influence upon the riboflavin or thiamine content of the liver. Animals maintained on pyridoxine- or pantothenic acid-free diets until very severe signs of deficiency had become manifest (after 49 to 60 days) showed no significant deviations in the riboflavin or thiamine concentration of the liver (Table II). Likewise, no change was observed in the riboflavin content of the liver in animals suffering from severe biotin deficiency or from vitamin A deficiency (Table II).

Thiamine Deficiency—The thiamine in the liver of the rats maintained on diets deficient in this vitamin decreased rapidly during the deficiency period (Table III). A depletion period of 24 to 28 days led to an almost complete exhaustion of thiamine in the liver, average values amounting to 0.2 γ per gm. (less than 10 per cent of the control level). In all thiamine-deficient animals the concentration of riboflavin in the liver was consistently higher than that of control rats receiving adequate amounts of thiamine. This increase was not significant during the first 13 days while the rats were still growing (Experiment 1). However, after 24 to 28 days the riboflavin concentration in the liver of thiamine-deficient rats reached values which were almost 50 per cent higher than those of a control group which received the same amount of riboflavin by stomach tube (Experiment 2). A similar increase in the riboflavin concentration of the liver was also found in thiamine-deficient animals which received riboflavin and other B vitamins in the basal diet (Diet 18A) (Experiment 4) or in Diet 20 (Experiment 5). When the control animals were restricted in their food consumption to that of the thiamine-deficient animals, no increase in the riboflavin concentration of the liver was observed (Experiment 3). Likewise, mice depleted in thiamine by the feeding of pyrithiamine showed a

TABLE III

Effect of Thiamine Deficiency upon Concentration of Thiamine and Riboflavin in Liver

Experiment No.	Diet	No of animals	Days on diet	Body weight		Thiamine per gm. liver		Riboflavin per gm. liver	
				Average	Range	Average	Range	Average	Range
				gm.	*gm.*	γ	γ	γ	γ
1	18, deficient	6	13	65	52– 73	1.3	0.9– 2.6	19.0	15.1–22.2
	18, control	3	13	73	69– 80	5.4	4.9– 5.8	16.0	15.2–17.6
2	18, deficient	18	24–28	50	35– 70	0.2	0.1– 0.3	30.0 ± 0.8	22 –36.8
	18, control	11	24–28	123	110–152	2.5	2.0– 2.6	21.8 ± 0.1	19.8–24.6
3	18, deficient	7	20	49	43– 57	0.6	0.3– 1.0	27.8	21.6–32.4
	18, control	5	20	103	88–113	9.3	7.6–12.8	23.1	21.0–25.4
	18, " paired feeding	7	20	68	55– 76	9.6	7.2–10.9	20.9	19.2–25.4
4	18A, deficient	8	25	55	45– 60	0.2	0.1– 0.3	31.5	26.5–34.6
	18A, control	5	25	155	138–194	10.7	8.1–12.6	24.4	22.9–27.6
5	20, deficient	10	29–32	54	46– 65	0.4	0.2– 0.7	29.5	24.2–36
	20, control	8	29–32	137	130–144	4.4	2.3– 5.7	25.5	22 –29
6	Pyrithiamine (mice)	3				2.2	1.9– 2.6	36.8	34 –41
	Control	3				9.2	4.9–16.1	30.5	29 –32

higher riboflavin concentration in the liver than did control animals which received large amounts of thiamine to counteract the effect of pyrithiamine (Experiment 6). When thiamine-depleted rats were given thiamine, the riboflavin concentration in the liver was found to decrease gradually during the following 2 to 4 days to the values of the control group, while the thiamine concentration in the liver increased within 2 to 4 hours to normal.

Riboflavin Deficiency—In contrast to the rapid loss of thiamine in the liver of thiamine-deficient rats, the decrease of riboflavin in the liver of riboflavin-deficient rats progressed at a slow rate. After 24 days of depletion the concentration of riboflavin was about 10 γ per gm. or slightly less than 50 per cent of that of the controls (Table IV). Even in moribund animals appreciable amounts of this vitamin were found; riboflavin concentrations lower than 6 γ per gm. apparently are incompatible with life.

TABLE IV

Effect of Riboflavin Deficiency on Concentration of Riboflavin and Thiamine in Liver

Diet	No. of animals	Days on diet	Body weight		Riboflavin		Thiamine	
			Average	Range	Average	Range	Average	Range
			gm.	*gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*
18, deficient	5	24	36	35– 39	10.3	8.8–11.2	12.0	9.7–15
18, control	8	24	84	62–106	19.5	11.6–24.8	5.1	3.9– 6.7
18, " paired feeding	5	24	66	62– 71	18.9	11.6–24.8	5.1	4.4– 6 3
18, deficient	5	25 (24 hrs. after 160 γ riboflavin)	48	42– 53	10.8	9.3–12.6	7.5	5.5– 9.8
18, "	4	26 (48 hrs. after 320 γ)	53	46– 59	15.8	14.8–17.3	7.3	4.0– 9.0
18A, deficient	10	27	52	44– 60	9.7	6.4–12.6	16.5	12.6–20.6
18A, control	5	27	155	138–194	24.9	22.9–27.6	10.7	8.1–12.6
18A, " paired feeding	5	27	99	96–103	16.0	14.4–18.6	8.6	5.7–12.2

The thiamine concentration in the liver of riboflavin-deficient animals was without exception higher than that of the control animals. In some experiments the values were twice those of the control animals which received the same amount of thiamine daily by stomach tube. It was likewise increased when the vitamins were fed in the basal diet (Diet 18A). The restriction of food consumption in control animals had no effect upon the thiamine level of the liver. The thiamine concentration of the livers of these animals was not significantly different from that of the control animals eating *ad libitum* (Table IV).

Another group of riboflavin-deficient animals was fed 160 γ of riboflavin daily after a depletion period of 24 days (Table IV). The riboflavin concentration in the liver increased slightly over a period of 24 hours; a significant rise was found only after 48 hours. In contrast, the thiamine concentration in the liver showed a considerable decrease during the first 24 hours.

Vitamin B Complex Deficiency—In these experiments animals were maintained on the vitamin B complex-deficient basal Diet 18 supplemented daily with 5 mg. of choline only. This depletion in B vitamins produced a rapid decrease in the thiamine concentration of the liver (Table V). After 30 days thiamine levels of 0.2 γ per gm. were found, values comparable to those observed in thiamine deficiency alone (Table III). The riboflavin concentration, however, decreased only slightly during the first 3 weeks and still averaged 15.1 γ per gm. after 30 days. In contrast, animals depleted in riboflavin alone showed, after 24 days, a much lower level of riboflavin; namely, 10.3 γ per gm. (Table IV).

TABLE V

Effect of Depletion in All B Vitamins (Except Choline) upon Concentration of Thiamine and Riboflavin in Liver

Diet 18	No. of animals	Days on diet	Body weight		Thiamine*		Riboflavin	
			Average	Range	Average	Range	Average	Range
			gm.	*gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*	*γ per gm.*
Deficient............	9	22	38	29– 47	0.4	0.2–0.9	18.6	12.2–25.6
Control............	3	22	90	87– 96	3.1	2.4–3.7	19.4	16.1–21.4
Deficient............	3	30	38	35– 40	0.2	0.2–0.3	15.1	12.1–17.6
Control............	2	30	137	135–138	2.4	2.3–2.4	21.5	20.8–22.2

* We are indebted to Dr. D. M. Tennent for these thiamine values.

DISCUSSION

The riboflavin concentration in the liver was found to be significantly increased in thiamine-deficient rats, and riboflavin-deficient rats had an increased concentration of thiamine in the liver. Furthermore, withdrawal of all B vitamins except choline resulted in a rapid loss of thiamine, but the level of riboflavin in the liver remained relatively stable for a long period. On the other hand, the level of thiamine and of riboflavin in the liver of animals severely depleted in pyridoxine, pantothenic acid, or biotin failed to show significant deviations from that of control animals receiving adequate amounts of these vitamins.

The increase in riboflavin in the liver of thiamine-depleted rats amounted to 50 per cent over that of controls, whereas the thiamine concentration in riboflavin-deficient livers was up to 100 per cent higher than in control animals. The increase of either thiamine or riboflavin was not due to inanition, since isocaloric feeding of the control animals failed to increase the vitamin content of the livers. Nor can the increased vitamin content be explained by a change in moisture content of the organ, since no significant differences in water content were observed between deficient and control animals. The relative size of the liver was considerably decreased in deficient animals; the ratio of 4.0 to 5.0 between liver weight and kidney weight in normal animals was reduced to 2.5 to 3.5 in severely deficient animals. However, diminution in liver size appears not to be responsible for the selective increase in thiamine or riboflavin, since a comparable shrinkage of the liver in rats severely depleted in other B vitamins was not associated with significant changes in thiamine or riboflavin content.

Our results do not support the postulation of Sure and Ford (7) that riboflavin deficiency might result from poor utilization of riboflavin in thiamine deficiency. On the contrary, our thiamine-deficient rats had more riboflavin available than controls, and the feeding with thiamine resulted in a dispersal of the excess riboflavin from the liver.

Ferrebee and Weissman (8) using adult rats did not observe a significant change in the riboflavin concentration of the liver of adult rats maintained on a thiamine-deficient diet for a period of 4 weeks. On the other hand, in a recent paper by Schweigert, McIntire, and Elvehjem (14) protocols are published from which it is evident that the thiamine concentration in the liver is appreciably higher in rats receiving 8 γ of riboflavin than in others receiving 60 γ of riboflavin per day. For instance, the average thiamine values reported on a high carbohydrate-low riboflavin diet are 14.5 γ per gm. of dry weight as compared to 7.6 γ on a high carbohydrate-high riboflavin diet, differences which are quite comparable to our findings.

SUMMARY

In young rats depletion in thiamine was found to increase the concentration of riboflavin in the liver above that of control animals. In riboflavin deficiency, the thiamine concentration of the liver was higher than in control animals.

The concentrations of thiamine and riboflavin in the liver of rats deficient in pyridoxine, in pantothenic acid, in biotin, or in vitamin A were not significantly different from those of litter mate controls maintained on adequate amounts of these vitamins.

The results are interpreted as evidence of an interdependence of thiamine and riboflavin.

We wish to express our thanks to Dr. D. W. Woolley of The Rockefeller Institute for Medical Research for the pyrithiamine-treated mice used in this study.

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THE EFFECT OF VITAMIN DEFICIENCY ON ESTRADIOL INACTIVATION BY LIVER*

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(Received for publication, March 22, 1944)

Several authors have investigated the rôle of liver in estrogen metabolism. Liver slices from dogs, rats, and mice have been found by Zondek and Sklow (1) and by Heller (2) to metabolize estrogens *in vitro*. Perfusion experiments on isolated organs have been performed by Israel *et al.* (3). Perfusion of estrone through a heart-lung preparation did not result in inactivation. However, when the liver was introduced into the circulatory path, estrogen was rapidly destroyed. Further evidence of the rôle of the liver in estrogen metabolism has been advanced by Biskind and Mark (4). They implanted a pellet of estrogen in the spleen of a castrate rat. No estrogenic effect was observed as long as the spleen remained connected with the portal circulation. However, when the spleen was transplanted and its venous blood flowed directly into the systemic circulation, the estrogenic effect of the implanted hormone became apparent.

Using a similar technique, Biskind and Biskind (5) and Biskind and Shelesnyak (6) demonstrated an interrelationship between vitamin B complex deficiency and the production of experimental estrus by estradiol. Biskind and Biskind implanted a pellet of estrogen in the spleen of castrate female rats maintained on a diet low in B vitamins. The implanted hormone was not inactivated, as shown by the occurrence of protracted estrus. Castrate female rats maintained on a complete diet, however, remained in anestrus after similar implantation. Biskind and Shelesnyak carried out another series of experiments in rats in which one ovary was removed and the other transplanted into the spleen. Thus all the blood flowing through the ovary entered the portal circulation. These animals showed signs of estrus when maintained on a diet deficient in the vitamin B complex, whereas the controls fed a normal diet failed to do so.

The present paper deals with the effect of deficiencies of individual known members of the B complex and of vitamin A on the ability of rat and mouse liver slices to inactivate estradiol.

Production of Deficiencies—The rats employed in studying the various

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* This work was carried out with the aid of a grant from the Nutrition Foundation, Inc.

deficiencies were males and females from two strains. They were all started when 21 to 28 days of age. In order to produce thiamine, riboflavin, pyridoxine, and pantothenic acid deficiencies, animals were placed on a purified vitamin B complex-free basic diet (Diet 18) consisting of casein (vitamin-free) 18 per cent, dextrose 68 per cent, salt mixture, U. S. P. XI No. 1, 4 per cent, hydrogenated vegetable fat (Crisco) 8 per cent, and cod liver oil 2 per cent. The animals were given a daily supplement, fed by stomach tube, of the following synthetic materials (values expressed in micrograms): thiamine 40, riboflavin 80, pyridoxine 40, calcium pantothenate 200, nicotinamide 500, and choline chloride 5000. In addition all animals received 5 mg. of α-tocopherol dissolved in ethyl laurate once a week. In order to produce any given deficiency, the respective vitamin was omitted from the daily supplement. The diets employed in producing biotin- and vitamin A-deficient animals, as well as other diets used for producing thiamine deficiency, are reported by Singher *et al.* (7). The estradiol-inactivating ability of the liver showed the same dependence on liver vitamin level irrespective of the diet employed in producing the deficiency.

Rats or mice were maintained on the respective diets until sacrificed. The animals were killed at various stages from the appearance of the first signs of deficiency until very severe symptoms were apparent. In several cases the animals were already near death due to the deficiency.

In experiments dealing with the level of riboflavin essential for estradiol inactivation large groups of animals were maintained on the deficient diet until gross signs of deficiency became apparent. Some of these animals were sacrificed immediately, while the other members of the groups were given 160 γ of riboflavin daily and sacrificed at various intervals until liver riboflavin values approached the normal.

Inactivation Technique—When the animals were sacrificed, the livers were immediately removed and sliced into Ringer-phosphate solution. Preliminary experiments with normal rats had demonstrated that 100 mg. of slices were just adequate for the inactivation of 3 γ of estradiol in the incubation period of 2 hours. This was considered the oxidative threshold for estradiol under our conditions. This amount of tissue was weighed into the main well of Warburg vessels which contained 2 cc. of Ringer-phosphate solution and 0.2 cc. of 20 per cent KOH in the center well. There was then added to the main vessel 0.01 cc. of a solution containing 3 γ of estradiol[1] with a Guthrie pipette controller and a 0.02 cc. pipette. The flasks were then incubated, with continuous shaking, at 37.5° for 2 hours and the respiration of the tissue observed. Only those experiments which showed actively respiring tissue were included in the final results. At the end of the 2 hour period the tissue was immediately removed from the vessel. The fluid in the Warburg vessels was then transferred and the vessels were rinsed several times with equal volumes of distilled water. The tissue, fluid, and washings were combined and placed in a boiling water bath for 10 minutes. The heat-inactivated material was then homogenized and made up to a volume such that 3 cc. could contain no more than 0.4 γ of estradiol.

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[1] We wish to express our thanks to Dr. Schwenk of the Schering Corporation, Bloomfield, New Jersey, for the generous gift of the estradiol employed.

Assay Method—This material then was injected into immature Sprague-Dawley female rats and assayed for estradiol content by the uterine growth method by the technique of Lauson *et al.* (8).

Vitamin Determination—For the determination of riboflavin 200 to 400 mg. of fresh liver were dried on filter paper, weighed, and homogenized with 0.25 M sulfuric acid. This was transferred quantitatively with more acid to a small beaker and hydrolyzed in boiling water for 20 minutes in the dark. The hot suspension was diluted and neutralized with a saturated solution of disodium phosphate. This was filtered in the dark and made up to a volume of 50 cc. The final dilution was read in the fluorophotometer against a standard of pure riboflavin according to the method of Hodson and Norris (9).

In the thiamine determinations sufficient fresh or frozen liver was employed to yield 5 γ of the vitamin, whenever possible, in the final aliquot. This tissue was homogenized in 0.1 N sulfuric acid and the pH then adjusted to 4 to 4.5. 300 mg. of taka-diastase were then added to this solution and the mixture incubated for 2 hours. This was then centrifuged, the precipitate washed with water, and the thiamine in the combined centrifugate and washings determined by the method of Hennessy and Cerecedo (10).

Results

The values reported in Tables I to IV for vitamin content are expressed as micrograms per gm. of wet weight of liver.

The plus and minus signs in the estradiol inactivation columns represent inactivation or the failure of inactivation of the added estradiol by liver slices. Plus signs represent an estradiol content, after incubation, of less than 0.9 γ. Minus signs represent material containing 2 γ or more of estradiol. Assays yielding 0.9 to 2 γ are given as (±) since they represent a diminution of at least 50 per cent in the original estrogenic activity.

As shown in Table I, the reduction of the riboflavin content of rat livers, which occurs after approximately 3 weeks on the deficient diet, is associated with a loss of the estradiol-inactivating ability. The animals showed, in the later stages of this deficiency, the gross morphological changes associated with riboflavin depletion. Further data on riboflavin-deficient animals are included in Table II. From the values in both tables, it is apparent that under these experimental conditions a riboflavin level of 13 to 14 γ per gm. of liver is essential for estradiol inactivation by liver slices. Further, the activity, presumably enzymatic, lost by depletion can be restored by the feeding of the vitamin. The restoration of activity in these deficient animals following the administration of riboflavin is relatively slow and parallels the slow rise in the liver riboflavin content.

The effects of thiamine deficiency (Table III) demonstrate the dependence of estradiol inactivation, by liver slices, on the liver content of this vitamin. With a method of thiamine determination by which there is no differentiation between the free and combined forms of the vitamin, it was found that the thiamine content would return to the normal values in a period of not more than 2 hours under our conditions. The estradiol-inactivating ability was restored in the same period. The data on vitamin B complex deficiencies in the rat (Table IV) reflect changes mainly in the thiamine content. The failure of the liver to inactivate estradiol appears to be due to the loss in thiamine. Mouse liver slices, from mice depleted in riboflavin, thiamine, or the whole vitamin B complex, react in an entirely analogous manner to that of the rats (Table V).

TABLE I

Ability of Liver Slices from Riboflavin-Deficient Rats to Inactivate Estradiol

Days on diet	Riboflavin		No. of rats	No. able to inactivate estradiol
	Average	Range		
	γ per gm.	*γ per gm.*		
12	17.8	15.4–19.4	5	5+
19	11.9	10 –14	4	4−
28	13.1	12.6–13.5	3	3−
35	12.6	10.7–15.2	3	3−
48*	15.4	15.3–15.4	2	2+
48	15.3		1	1+
48	8.8	7.4–10.2	2	2−
57			3	3−
61*			4	3−, 1+
67			5	4−, 1±
Controls fed riboflavin				
12–48	23.2	18.6–28.8	9	9+
57–67			9	9+

* Received 2.5 γ of riboflavin daily.

TABLE II

Rat Liver Riboflavin Level Essential for Estradiol Inactivation

Experiment	Days on riboflavin-free diet	Days receiving riboflavin supplement before sacrificing	Total riboflavin fed over depletion period	Riboflavin in liver		No. of rats	No. able to inactivate estradiol
				Average	Range		
			γ	γ *per gm.*	γ *per gm.*		
A	23 (Controls)	0	0	19.0	18.2–19.9	2	2+
	23	0	0	13.3	12.5–14.1	2	2−
	23	2	320	12.2	11.1–13.3	2	1+, 1−
	23 (Controls)	6	960	20.9	20.3–21.5	2	2+
	23	6	960	13.9	12 –16.7	3	3+
B	24 (Controls)	0	0	20.3	16.4–24.8	3	3+
	24	0	0	10.3	8.8–11.2	5	5−
	24 (Controls)	1	160	21	18.2–23.8	2	2+
	24	1	160	10.8	9.3–12.6	5	5−
	24 (Controls)	2	320	20.6	20.2–21.0	2	2+
	24	2	320	15.1	14.8–17.3	5	5+
	24 (Controls)	2	320	22.6		1	1+
	24	2	320	15.3	12.7–17.9	2	1+, 1±
C	42 (Controls)	0	0	24.2	24.1–24.2	2	2+
	42	1	160	7.1	6.4– 7.8	3	3−
	42 (Controls)	4	640	25.4	23.5–27.4	2	2+
	42	4	640	12.7	12.5–12.9	2	1−, 1+
	42	4	640	17.7		1	1+

TABLE III

Ability of Thiamine-Deficient Rat Liver Slices to Inactivate Estradiol

Days on diet	Thiamine		No. of rats	No. able to inactivate estradiol
	Average	Range		
	γ *per gm.*	γ *per gm.*		
13	1.3	0.86–2.6	6	4−, 2+
28			4	4−
26	0.4	0.0 –0.8	4	3−, 1±
26	0.28	0.16–0.46	4	4−
24			2	2−
32			2	2−
30	0.34	0.23–0.43	4	4−
Controls fed thiamine				
13–30	3.9	2.0 –5.8	10	10+
24–32			8	8+

In a study of the vitamins that were found to be without effect on estradiol metabolism, under those conditions, animals were kept as long as possible, in many cases until near death, on the deficient diet. With these "ineffective" vitamins (Table VI) animals, especially in late stages of deficiency, showed, markedly, the gross symptoms associated with the

TABLE IV

Ability of Liver Slices from Vitamin B Complex-Deficient Rats to Inactivate Estradiol

Days on diet	Thiamine*		Riboflavin		No. of rats	No. able to inactivate estradiol
	Average	Range	Average	Range		
	γ per gm.	γ per gm.	γ per gm.	γ per gm.		
22	0.4	0.2–0.9	18.9	12.2–22	9	5–, 4±
30	0.2	0.2–0.3	15.1	12.1–17.7	3	2–, 1±
Controls fed all B vitamins						
22–36	3.6	2.3–8.3	21.5	16.1–27.8	6	6+

* We are indebted to Dr. D. M. Tennent for these thiamine analyses.

TABLE V

Inactivation of Estradiol by Mouse Liver Slices

Deficiency	Days on diet	No. of animals	No. able to inactivate estradiol
Riboflavin	15	2	2+
Thiamine	15	2	2–
Vitamin B complex	15	3	2+, 1–
Riboflavin	20	3	2–, 1+
Thiamine	20	2	2–
Controls (receiving all B vitamins)	15–20	5	5+

TABLE VI

Inactivation of Estradiol by Liver Slices from Rats with Various Deficiencies

Deficiency	Days on diet	No. of rats	No. of rat livers able to inactivate estradiol
Pyridoxine	46– 49	13	13+
" controls	46– 49	5	5+
Pantothenic acid	35– 43	22	19+, 3±
" " controls	35– 43	11	10+, 1±
Biotin	35– 68	7	5+, 2±
" controls	35– 48	4	4+
Vitamin A	48–136	11	10+, 1±
" " controls	48–136	11	11+

particular deficiency. In this category of "ineffective" vitamins are pyridoxine, pantothenic acid, biotin, and vitamin A (Table VI).

Both male and female rats have been included in the various groups of deficient animals. These have reacted similarly in all cases, indicating that sex does not play any essential rôle in estradiol inactivation in liver slices. There does not appear to be any difference in estradiol-inactivating ability in liver within 26 to 154 days.

The effect of restricted protein intake upon estradiol-inactivating ability is under investigation as well as a more detailed investigation of the enzyme systems involved.

DISCUSSION

Our results clearly demonstrate that, of the vitamins tested, riboflavin and thiamine are essential in the metabolism of estradiol by liver slices. The inactivation of estradiol is dependent upon the concentration of these vitamins in the liver.

In view of the work of Westerfeld (11) on chemical oxidation of estrogens and of his work (12) on its oxidation by tyrosinase, it seems possible that these vitamins may be involved in estrogen metabolism through their rôle as members of an oxidative enzyme system. The recent report by Schiller and Pincus (13) of the recovery of various estrogenic materials of lower potency after the perfusion of estradiol through heart-lung-liver preparations is not inconsistent with such a hypothesis. Any oxidation or reduction product of the estrogenic material, whether active or inactive, would fit equally well into any scheme involving the vitamins as members of such a metabolic system.

The presence of excess free estrogen may be associated with a number of clinical syndromes. A relation of those manifestations to nutritional deficiency is supported by the work of Biskind and our findings. In this connection it is interesting to note that Ashworth and Sutton (14) have reported that estrogens either increase the demand for or suppress the utilization of members of the vitamin B complex in human patients. Biskind, Biskind, and Biskind (15) have recently presented evidence that certain gynecological conditions are associated with nutritional deficiencies and respond to vitamin B complex therapy.

SUMMARY

Liver slices from riboflavin- and thiamine-deficient rats are unable to inactivate estradiol under conditions in which slices from controls on the same diets, but receiving adequate amounts of these vitamins, possess this activity. This loss of inactivating ability parallels the change of riboflavin and thiamine content in the liver. Pyridoxine, pantothenic acid,

biotin, and vitamin A deficiencies have no effect on estradiol inactivation under the same conditions.

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THE PROTEIN-FORMALDEHYDE REACTION

I. COLLAGEN

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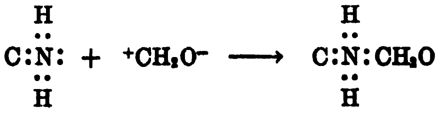
(Received for publication, March 8, 1944)

A careful study of the literature relating to the reaction between the fibrous proteins and formaldehyde suggests that some divergence of opinion exists. Harris (11) and Birch and Harris (4) in 1930 showed that the titration of amino acids, with hydrochloric acid in the presence of formaldehyde, is not affected, while the titration with sodium hydroxide is markedly affected. Harris explained this phenomenon as the repression of acidic groups upon acid titration and the repression of basic groups upon alkaline titration.

Using the zwitter ion concept of Bjerrum (5), we would then not expect formaldehyde addition to affect in any way the acid titration of a protein, while we would expect it to influence the alkaline titration, since in the alkaline zone the formaldehyde undoubtedly reacts in some manner with the available free and uncharged amino groups. Thus, this concept would lead us to expect little or no shift in the isoionic point of the protein upon treatment with formaldehyde.

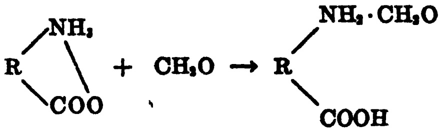
Tomiyama (26) believes the anionic form of the amino acid reacts with the formaldehyde. He also considers the protein-formaldehyde reaction in terms of the electronic theory. He pictures the formaldehyde as a dipolar molecule, $^{+}CH_2O^{-}$, and since the amino or imino group of the anionic form of the amino acid has 2 unshared electrons, the two components react to give the accompanying formula.



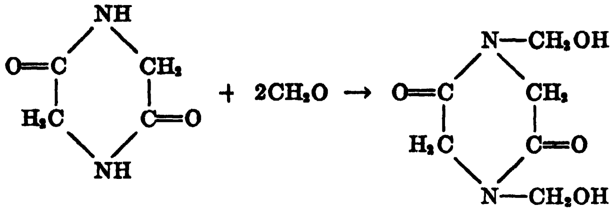
$$C:\overset{\displaystyle H}{\underset{\displaystyle H}{\overset{\cdot\cdot}{\underset{\cdot\cdot}{N}}}}: + {}^{+}CH_2O^{-} \longrightarrow C:\overset{\displaystyle H}{\underset{\displaystyle H}{\overset{\cdot\cdot}{\underset{\cdot\cdot}{N}}}}:CH_2O$$

Levy and Silberman (15) have shown mathematically from their studies that 2 molecules of formaldehyde combine with 1 molecule of amino acid. Bergmann *et al.* (3) have isolated a triformyl compound and further shown that the triformyl derivative changes to the monoformyl upon addition of alkali.

Reiner and Marton (16) postulated the following reaction between protein and formaldehyde,



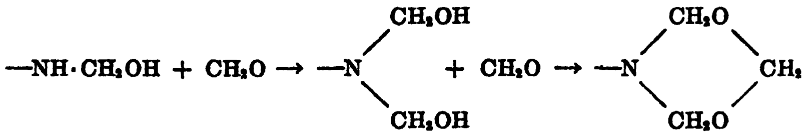
the aldehyde being held to the amino group by secondary valence. Einhour (8) showed that the acid amides fix formaldehyde and suggested the following reaction, $R{-}CONH_2 + CH_2O \rightarrow R{-}CONH\cdot CH_2OH$. Cherbuliez and Fier (7), and later Bergmann, found that diketopiperazines react with formaldehyde, taking up 2 molecules of the aldehyde.



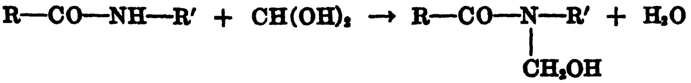
Levy and Silberman (15) have taken exception to the interpretations of Tomiyama, maintaining that he made no distinction between amino and imino groups. Balson and Lawson (2) have suggested that the number of formaldehyde groups which can be introduced corresponds to the number of hydrogen atoms attached to the nitrogen atom and have therefore proposed Reactions 1, 2, and 3.

(1) $=NH + CH_2O \rightarrow =N\cdot CH_2OH$

(2) $-NH_2 + CH_2O \rightarrow -NH\cdot CH_2OH$

(3) 

Stiasny (17) has suggested that formaldehyde reacts with gelatin in possibly two ways, in one, with the basic groups, changing them to neutral ones, and in the other, with the imino groups of the peptide linkage. He suggests that the first reaction proceeds through an intermediate formation of a triformyl derivative which then changes to the monoformyl. In the second reaction, Stiasny postulates a binding of the formaldehyde with the weakly basic imino groups, *forming* methylol compounds,



$$R{-}CO{-}NH{-}R' + CH(OH)_2 \rightarrow R{-}CO{-}\underset{\displaystyle CH_2OH}{\underset{|}{N}}{-}R' + H_2O$$

Stiasny further suggests that the free amino groups of the gelatin react rapidly, while the peptide groups only do so gradually. He believes that the action of the formaldehyde on the basic groups is such that not only the acid and base fixation capacity is influenced but also that of the fixation of tanning materials and dyes.

Since 1936 a number of papers, dealing with the reaction of the fibrous proteins and formaldehyde, have appeared in the literature. Theis and Schaffer (24) studied the collagen-formaldehyde reaction through a quantitative measurement of the breakdown of the internal structural forces as indicated by the change in "shrinkage" temperature. This work was followed by several additional papers (23, 22, 20, 19) relative to the actual fixation of formaldehyde by collagen. In 1939 Highberger and Retzsch (12) and in 1940 Highberger and Salcedo (13) investigated this reaction in a comprehensive manner. Bowes and Pleass (6), Holland (14), and Gustavson (10) have also studied this reaction in recent years.

EXPERIMENTAL

Specially prepared collagen material was used for the experiments. The preparation of the collagen has been previously described (21). 2 gm. samples of the collagen were placed in bottles together with 200 ml. of 0.1 N KCl solution made 1 per cent with respect to formaldehyde and then the series of samples was adjusted to definite hydrogen ion concentrations with either hydrochloric acid or sodium hydroxide. The range covered was from pH 1.0 to 13.0. The bottles and contents were placed in a thermostat maintained at 20° for 72 hours. At stated periods, the samples were agitated in order to promote equilibrium. After the 72 hour period the pH at equilibrium was determined by means of a Beckman glass electrode assembly; the protein material was removed and pressed several times between blotting paper at 10,000 pounds per sq. in. It has previously been shown that such pressure removes the free water and any free electrolyte for all practical purposes. We, therefore, have assumed that all free formaldehyde is correspondingly removed, leaving behind only that which is firmly bound to the protein itself. After being pressed, the protein-formaldehyde compound was allowed to dry in air, and was then ground in a small Wiley mill to a 60 mesh powder. The material was then ready for analysis for nitrogen, for bound acid or base, and for fixed formaldehyde. The method used for the determination of formaldehyde is that of Highberger and Retzsch (12) described elsewhere. The methods used for the nitrogen and for bound acid or base have been previously described (21).

Fig. 1 shows the data obtained. The series of curves may be interpreted as follows:

Curve A represents the regular acid or base fixation for the particular

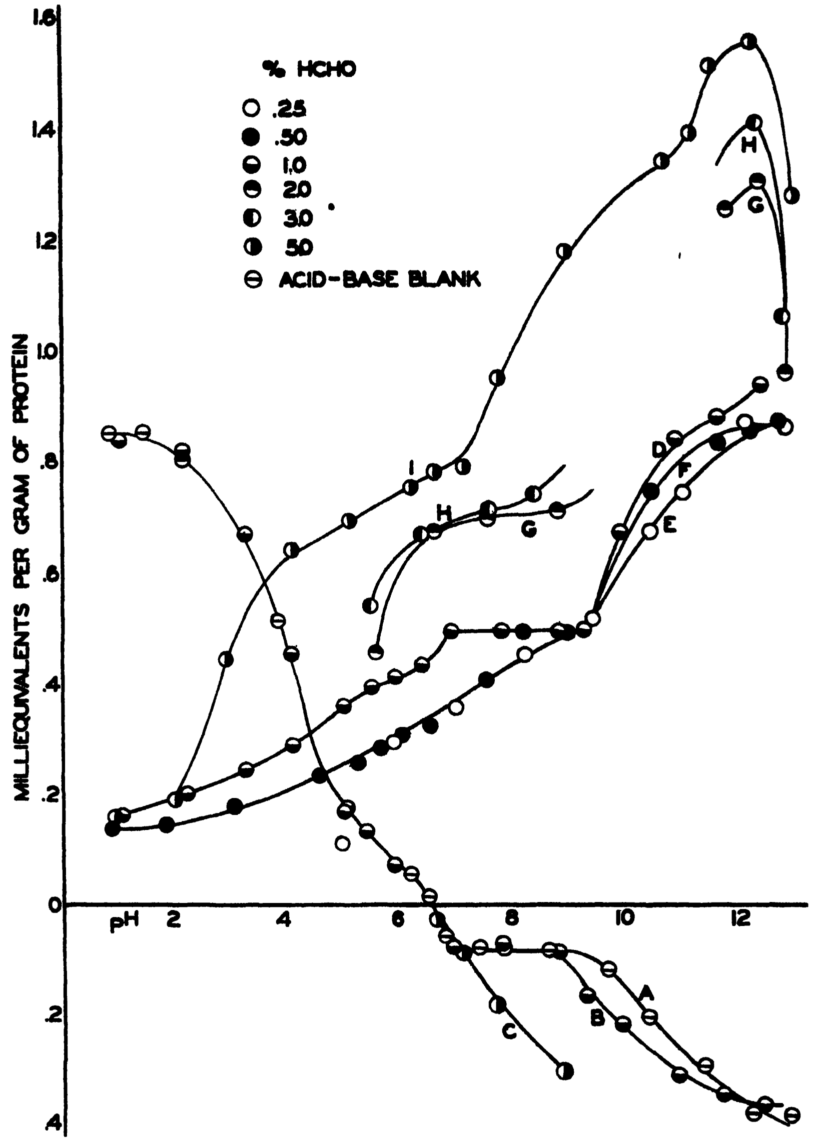


Fig. 1. Showing the acid, base, and formaldehyde bound by collagen treated with different amounts of formaldehyde over a wide pH range.

native collagen used, showing a maximum acid fixation of 0.87 milliequivalent per gm. of protein, a maximum base fixation of 0.38 milliequivalent, an isoionic point at pH 6.5, a plateau in the pH range 7.0 to 9.0 indicative of the back titration of histidine and of such α-amino groups as may be present, and a sharp point of inflection at pH 10.0, beginning the back titration of the ϵ-amino groups of lysine. This curve is identical in trend with those given in earlier work with the exception of the isoionic point which in this case is somewhat more acid.

Curve B represents the titration curve of the collagen-formaldehyde compound formed by the reaction of the collagen in a 1 per cent formaldehyde solution. This curve is identical with Curve A in the pH range 0.8 to 9.0. There is no indication of a shift in isoionic point. In the pH range 9.0 to 11.0 more base is fixed than is the case for the native collagen, indicative of the reaction between the ϵ-amino groups of lysine and formaldehyde. Curves A and B merge at pH 12.0 and approach a maximum value.

Curve C represents the acid or base bound by the collagen-formaldehyde compound formed in a 5 per cent formaldehyde solution. This curve is also identical with that of Curve A in the pH range 0.8 to 7.0 and definitely shows no shift in the isoionic point of the collagen. However, owing to the large excess of formaldehyde present during the reaction, more base is fixed in the pH range 7.0 to 9.0.

Curve D represents the formaldehyde fixed by collagen in a 1 per cent formaldehyde solution over a wide pH range. These data indicate about 0.15 milliequivalent of formaldehyde fixed at pH 1.0, thereafter increasing almost as a straight line function to 0.43 milliequivalent at pH 6.5 or at the isoionic point. At the isoionic point, there is a very definite break in the curve, the fixed formaldehyde increasing to approximately 0.5 milliequivalent and then remaining essentially constant from pH 7.0 to 9.5. At pH 9.5 there appears another break, the fixed formaldehyde increasing sharply at this point and continuing up to pH 11.5, at which point approximately 0.87 milliequivalent of aldehyde is bound. An apparent break in the curve occurs at pH 11.5, indicative of another reaction.

Curve E represents the formaldehyde fixed by collagen from a 0.25 per cent aldehyde solution. The curve shows a marked break at pH 9.5 and only a slight indication of a plateau region, but gives approximately the same aldehyde fixation at pH 12.5 as does Curve D.

Curve F represents the formaldehyde fixed by collagen from the 0.5 per cent solution. This curve shows a slightly lower aldehyde fixation in the acid zone compared with Curve D, a plateau in the pH zone 8.0 to 9.5, and approximately the same aldehyde fixation as is shown by Curve D at pH values greater than 9.5.

Curves G, H, and I represent formaldehyde fixation by collagen at the higher concentrations of formaldehyde; *i.e.*, the 2, 3, and 5 per cent formaldehyde solutions. Curves G and H show a definite point of inflection and even indications of a plateau in the pH range 6.5 to 8.5. These curves show a decided increase in aldehyde fixation at pH 12.0. Curve I shows a much greater aldehyde fixation in the acid zone, with a definite point of inflection at pH 7.0 and a maximum value of 1.6 milliequivalents of formaldehyde fixed at pH 12.0. Curves G, H, and I all show a definite decrease in aldehyde fixation at pH values greater than 12.0, possibly due to decrease in formaldehyde concentration because of the Cannizzaro reaction of formaldehyde itself at strong alkaline reactions.

DISCUSSION

Highberger and Retzsch, in explanation of their data, claim it is significant that the break, at pH 7.0 to 8.0 in their pH formaldehyde fixation curves, occurs at a formaldehyde fixation slightly over 0.4 mM per gm. of collagen. This value, they claim, is close to the amount of lysine believed to be present in collagen. These investigators state that this particular break represents the equivalence point in the reaction of 1 molecule of formaldehyde with each free amino group provided by the lysine residues and, therefore, *a priori* this is indicative that only the undissociated amino groups are involved in the reaction. They further postulate that the increase in formaldehyde fixation at pH values greater than 8.0 represents fixation with the stronger basic guanidino groups of arginine. This argument is advanced in spite of the fact that the pK_3 values of arginine and lysine are 12.5 and 10.5 respectively. They also point out that greater concentrations of formaldehyde cause a reaction between the excess formaldehyde and the imino linkages owing to their lesser basicity.

The experimental data given in this paper are not in line with those obtained by either Highberger and Retzsch or by Bowes and Pleass. Highberger and Retzsch show a possible but indefinite plateau zone in the pH range 7.0 to 8.0, while the writer shows a clearly defined plateau in the pH zone 6.9 to 9.4. The explanation given by Highberger and Retzsch is that at the particular break in the curve the ε-amino group of lysine has completely reacted with formaldehyde. The data given herein do not in any way support such an interpretation. The data given by Bowes and Pleass show a well defined maximum at pH 1.5, a minimum at pH 3.5, a constant fixation or plateau region at pH 5.0 to 11.0, a slight depression in fixation at pH 11.0, which they claim is real, and a sharp increase in fixation at pH 12.0. The data obtained by Highberger and Retzsch and by Bowes and Pleass are quite different from those given by us. The difference is due undoubtedly to the experimental methods used. After treating the collagen with formaldehyde, Highberger and Retzsch thoroughly washed the collagen-formaldehyde compound either with water or with dilute sodium bisulfite solution. The writer believes that this washing gave rise to erroneous and erratic results and that the final picture as obtained by Highberger and Retzsch does not represent the true one.

It must be borne in mind that the protein-formaldehyde reaction is a reversible one, the protein-formaldehyde compound being readily affected by any changes in hydrogen ion concentration, formaldehyde concentration, or other external conditions. That this is true can be readily seen from Table I, which shows the change in fixed formaldehyde of the collagen-formaldehyde compound formed in a 1 per cent formaldehyde solution at pH 11.0, (*a*) when it is placed in water at pH 8.0, (*b*) when it is placed in water at pH 2.0, and (*c*) when it is heated at 105° for 12 hours. These data show definitely that the collagen-formaldehyde compound is to a large extent reversible. Therefore, washing the compound after treatment with water can only yield erroneous values for fixed formaldehyde.

As an interpretation of the data given in Fig. 1, the writer suggests that in the pH range 1.0 to 6.4 the formaldehyde reacts with the slightly basic imino groups present in the peptide chains in such a manner as to form linkages or bonds between the polypeptide chains.

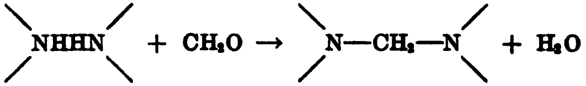
TABLE I

Formaldehyde Fixation Reversibility

	CH_2O*	H^+ or OH^-*
Treated at pH 11.0†	0.85	−0.33
" " " 11.0, then placed in water at pH 8.0	0.47	−0.11
" " " 11.0, " " " " " " 2.0	0.29	+0.94
" " " 11.0, " heated at 105°	0.70	−0.34

* 1 per cent CH_2O at pH 11.0 for 72 hours.

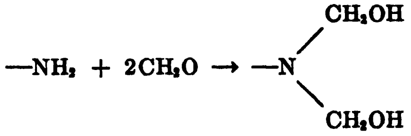
† Millimoles of CH_2O, H^+, or OH^- fixed per gm. of collagen.



$$\rangle NHHN \langle + CH_2O \rightarrow \rangle N{-}CH_2{-}N \langle + H_2O$$

In this manner additional cohesive bonds or bridges are built up, thus giving increased resistance to contraction or shrinkage. At pH 6.4, formaldehyde reacts with histidine, giving rise to the plateau zone at pH 6.9 to 9.4. The writer is well aware that there exists some difference of opinion whether the imidazole group of histidine reacts with formaldehyde (9). However, the present investigation appears to lend support to the view that such a reaction may take place, since in this particular pH range histidine is normally titrated back, as can be seen from a study of the acid-base binding data for collagen. As the pK_3 value of lysine is approached, we might expect the ε-amino group of lysine as it changes from $—NH_3^+$ to $—NH_2$ to react with formaldehyde. This appears to be the fact, as a study of Curve D, Fig. 1, shows that at pH 9.2 increasing formaldehyde fixation occurs. This increase is positive and approaches a definite point of inflection at pH 11.5. The increase in formaldehyde fixation between pH 9.2 and 11.5 is about 0.40 mM per gm. of collagen and approximates the lysine content (0.38 mM per gm.) of collagen. Such data and their interpretation indicate that the very basic guanidino group of arginine does not react to any great extent in the pH range studied. The writer believes, however, that if it were possible to study the reaction of collagen with formaldehyde at pH values greater than 12.0 we would find a further increase in formaldehyde fixation; *i.e.*, a binding with the guanidino group of arginine. The Cannizzaro reaction prevents such an investigation.

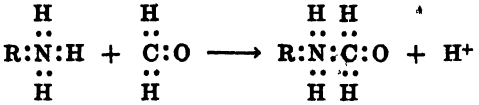
Curve I (Fig. 1) represents data secured when a large excess of formaldehyde is present during the reaction. These figures show that in the pH range 1.0 to 7.0 formaldehyde fixation is increased and must be due to a mass action effect, the aldehyde in all probability combining with a greater number of the weakly basic imino groups of the polypeptide chain. In the pH range 7.0 to 12.0, about 0.80 mM of formaldehyde has become fixed. This value represents approximately twice the lysine content of collagen and thus leads us to believe that 2 molecules of formaldehyde are fixed by each undissociated amino group of lysine,



$$—NH_2 + 2CH_2O \rightarrow —N(CH_2OH)_2$$

forming a dimethylol compound. Curves G and H, representing data for the collagen-formaldehyde compound formed upon treatment of collagen with 2 and 3 per cent formaldehyde solution, show in general the same trend. These two curves merge with Curves D and F at pH values less than 6.0, show a plateau at pH 6.5 to 9.0, and a sharp increase in formaldehyde fixation up to pH 12.0. Curve E represents data for the collagen treated with 0.25 per cent formaldehyde solution and shows only a slight indication of a plateau zone, but there is a definite indication of reaction of formaldehyde with histidine. Curves D, E, and F show approximately the same trend and amount of formaldehyde fixed in the pH range 9.5 to 12.0, indicative of a stoichiometric chemical reaction; *i.e.*, the fixation of 1 mole of formaldehyde with each ε-amino group of lysine.

In previously published work, Theis *et al.* (25) have postulated that, in the pH range 1.0 to 6.0, formaldehyde binds with the weakly basic imino groups of the polypeptide chains and not with the ε-amino group of lysine. It is certainly to be expected that with this preparation of collagen, having an isoionic point of 6.4, the basic groups would exist for the most part in the charged state at any pH value less than 5.0. Under such conditions, the basic groups of lysine and arginine would exist in the charged ionic form and the electronic pair of the nitrogen atom would not be available for formaldehyde fixation since it already is coordinated with the hydrogen atom. Thus the accompanying reaction should not take place, since in that



$$\mathrm{R{:}\underset{\underset{H}{\cdot\cdot}}{\overset{\overset{H}{\cdot\cdot}}{N}}{:}H + \underset{\underset{H}{\cdot\cdot}}{\overset{\overset{H}{\cdot\cdot}}{C}}{:}O \longrightarrow R{:}\underset{\underset{H}{\cdot\cdot}}{\overset{\overset{H}{\cdot\cdot}}{N}}{:}\underset{\underset{H}{\cdot\cdot}}{\overset{\overset{H}{\cdot\cdot}}{C}}{:}O + H^+}$$

case the ability to bind H^+ ions would be affected. A study of Curve A of Fig. 1 shows that the H^+ ion-binding capacity of collagen is unaffected by formaldehyde fixation. The preceding interpretation is supported by Atkin (1). In a discussion of his investigation dealing with the deamination of collagen, he states, "As a consequence we should expect the part of the curve corresponding to the back titration of the basic groups of lysine to disappear. This is between pH 9.0 and 10.0 and it is evident that in deaminized collagen this part of the curve has disappeared." In Curves D, E, and F of Fig. 1, the part of the curves representing the back titration of the lysine has not disappeared.

Theis and Esterly (20) have in the past used "shrink temperature" as a criterion for protein stabilization. "Shrink temperature" has been defined as the point at which the increasing disruptive tendencies exceed the diminishing cohesive forces; thus the "shrink temperature" is actually a measure of the structural strength of the collagen expressed in arbitrary units. Since x-ray data for collagen have shown that this protein exists in the native state as an extended polypeptide chain, it is evident that the chain may contract upon itself. Collagen in the moist state shows a shrinkage temperature of approximately 58°. If the collagen is treated with reagents which enter into combination with its reactive groups, this shrinkage temperature may decrease or increase. Collagen treated with formaldehyde at various pH values shows an increase in shrinkage temperature at practically all pH values. Fig. 2 shows such data for this particular collagen. Curve A represents the shrinkage temperature of native collagen, merely treated with aqueous acid or alkali. This curve indicates a shrinkage temperature of 57–58° in the pH range 6.0 to 9.5. At pH values less than 6.0 or greater than 9.5 decreased structural stability is evident. Curve B represents data for collagen treated at various hydrogen ion concentrations but in this case the acid or base solutions contained 0.5 per cent of formaldehyde. A striking difference is apparent. At all pH values the shrinkage temperature has definitely increased. This increase is particularly notable at pH values greater than 4.0. There are definite points of inflection at pH 7.0 and pH 8.0. Curve C represents data for a 1 per cent formaldehyde solution, while Curve D is that for a 5 per cent formaldehyde solution. Curve C shows a slight increase in shrinkage temperature over that shown by Curve B and in line with the curves given in Fig. 1. In the pH range 1.0 to 6.0, Curve D shows a decided increase in structural stability over that shown in either Curve B or C, again in line with formaldehyde fixation data given in Fig. 1. It is to be particularly noted, however, that in the zone pH 8.0 to 12.0 all three curves merge. Such trends would seem to indicate two different and distinct chemical reactions, the one taking place over practically the whole pH range being especially noticeable on the acid side of the isoionic point, and the other taking place at pH values greater than 8.0. This series of curves for shrinkage temperature lends support to the suggestion that, in both the acid and alkaline zones, it is the reaction of formaldehyde with the weakly basic imino groups of the polypeptide chains that gives to the collagen its thermolability as measured by the shrinkage temperature. In the alkaline zone, the formaldehyde undoubtedly binds with the free basic groups of lysine in addition to the imino groups.

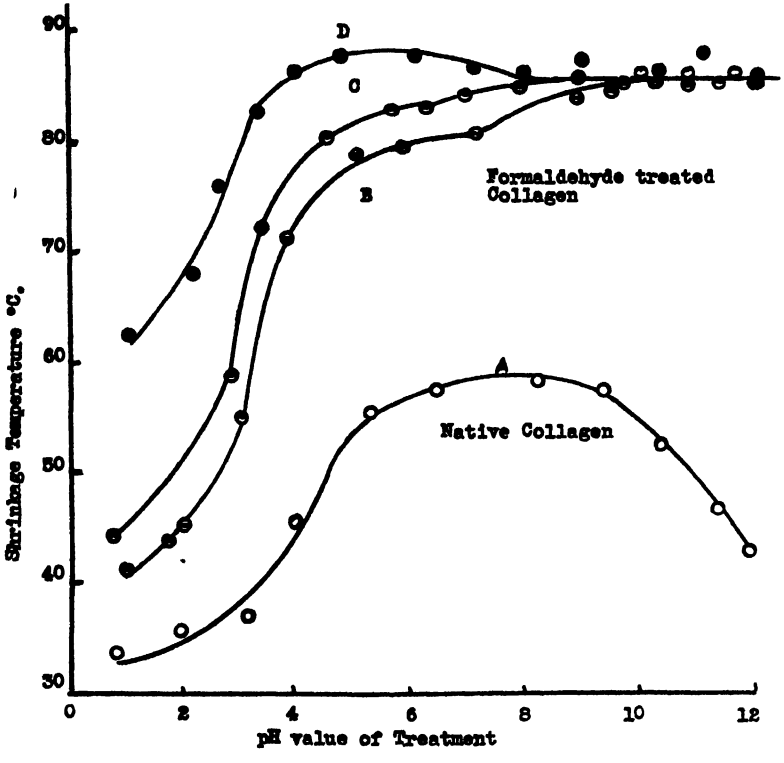


FIG. 2. Comparison of the shrinkage temperature of native collagen with that of formaldehyde-treated collagen.

SUMMARY

The collagen-formaldehyde reaction has been discussed in detail. It has been shown that the fixation of formaldehyde with collagen in no way affects the acid-binding capacity of collagen but does affect the base-binding capacity. No shift in the isoionic point could be demonstrated as due to formaldehyde fixation. Correlation between data for shrinkage temperature and formaldehyde fixation is shown.

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THE PROTEIN-FORMALDEHYDE REACTION

II. WOOL

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(Received for publication, March 9, 1944)

In Paper I (4) the collagen-formaldehyde reaction has been discussed. In that work it was shown that formaldehyde fixation with collagen in no way altered the acid-binding capacity of the protein or the isoionic point.

In 1942–43, Theis and Jacoby (5, 6) discussed the acid-base-binding of collagen and for that work used a technique which they believed gave a true picture of the nature of the acid and base binding of this particular fibrous protein. This work was then extended to cover the acid and base binding of collagen in the presence of formaldehyde.

Steinhardt and Harris (3) in 1940 studied the combination of wool proteins with acid and base. For this study they used a highly purified wool in contact with hydrochloric acid and with potassium hydroxide. For determining the acid or base bound, titration and potentiometric methods were used. They found the maximum acid bound to be 0.82 mM per gm. and the maximum base bound to be greater than 0.78 mM. They found that when salt is present the amount of acid or base bound changes with pH gradually, that there is no wide zone in which combination fails to take place, and that the point of zero combination occurs sharply at approximately pH 6.4.

EXPERIMENTAL

For the present study wool, thoroughly degreased through many changes of acetone, washed, pressed free from water, and then again dehydrated by acetone, was used.

2 gm. samples of the purified wool were placed in small bottles and 200 ml. of various concentrations of hydrochloric acid or potassium hydroxide solution, 0.1 N with respect to KCl, were then added. The concentrations were such that at equilibrium the pH values would vary between pH 1.0 and 12.0. The bottles and contents were then placed in a thermostat maintained at 20° for 72 hours. After equilibrium had been attained, the pH was measured by means of a Beckman glass electrode assembly. The wool samples were then pressed several times at 10,000 pounds per sq. in. in a Carver press. After being pressed, the wool samples were air-dried, ground in a small Wiley mill, and were then analyzed for nitrogen, and

acid and base bound. The methods of analysis have been given elsewhere (5). The data are shown in Curve A of Fig. 1 and represent the acid- and base-binding capacity of the purified wool.

In another series of experiments, 2 gm. samples of the purified wool were placed in 200 ml. of various concentrations of hydrochloric acid or potassium hydroxide solution made 0.1 N with respect to KCl and 1.0 per cent with respect to formaldehyde. In all other respects this series of experiments was identical with those described in the previous paragraph. In addition to the analyses for nitrogen and for acid or base bound, fixed formaldehyde was also determined by the method described by Highberger and Retzsch (1). These data are shown in Curves B and C of Fig. 1.

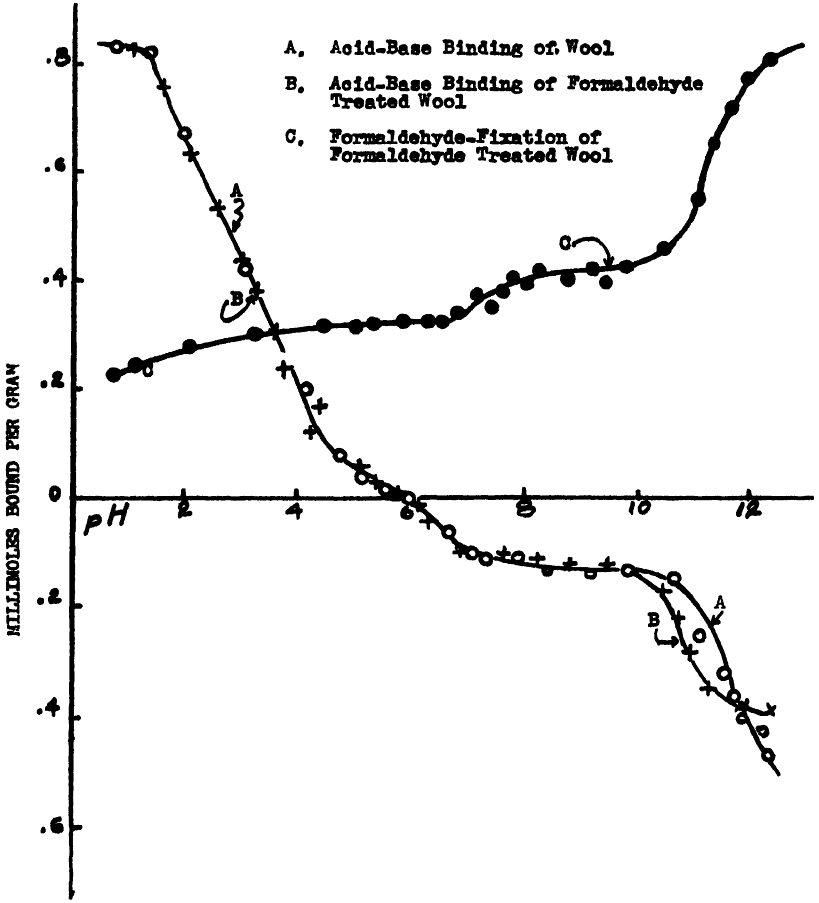


FIG. 1. The acid- and base-binding capacity of purified wool and the acid, base, and formaldehyde fixation of formaldehyde-treated wool.

DISCUSSION

Fig. 1 pictures the complete data for the acid- and base-binding capacity of purified wool, the acid- and base-binding capacity of formaldehyde-treated wool, and the formaldehyde fixation by wool over a wide pH range. The data may be interpreted as follows:

Curve A represents the normal titration curve of wool keratin and is similar to curves obtained by Steinhardt and Harris. This curve shows a maximum acid fixation of about 0.83 milliequivalent per gm. of protein at pH 1.0, an isioinic or zero-binding point at pH 6.1, a plateau region at pH 7.0 to 9.5, binding approximately 0.10 milliequivalent of base in this range, a sharp point of inflection at pH 10.5, and at pH values greater than 10.5 a significantly increased base-binding power.

Curve B represents the data for the formaldehyde titration curve. This curve is identical with Curve A in the pH range 1.0 to 9.5. At pH values greater than 9.5, slightly more base is bound. This curve shows an indication of a maximum base binding at pH 12.0. Owing to the tyrosine and cystine content of keratin, complications occur in the high alkaline zone and somewhat unsatisfactory values for maximum base fixation are obtained. For this reason more alkaline solutions were not used in this investigation.

Curve C indicates the formaldehyde fixed over the pH range studied. Up to and including pH 6.0, 0.32 milliequivalent of aldehyde is bound. At pH 6.0 an increased binding takes place, rising to 0.4 milliequivalent at pH 7.0, then remaining essentially constant until pH 10.0 is reached. At pH values greater than 10.0, a definite increase in aldehyde fixation occurs, approaching a maximum value of 0.82 milliequivalent at pH 12.5.

Steinhardt and Harris (3) list the reported acidic and basic amino acids of wool and these data are shown in Table I.

The sum of the arginine, lysine, and histidine equals 0.856 mM per gm. and the maximum acid bound shown in Curve A is 0.83 mM, well in line with the calculated values. This value compares extremely well with that of 0.82 mM obtained by Steinhardt and Harris using an entirely different method. The data in the pH zone 7.0 to 10.0 are also well in line with the results obtained by Steinhardt and Harris. The point for zero binding occurs at pH 6.1 as against the value 6.4 obtained by Steinhardt and Harris.

Since the data shown in this paper were taken only in the pH range 1.0 to 12.0, the maximum base-binding capacity cannot be determined from the curves in Fig. 1. However, up to pH 12.0, the alkali binding is of about the same order as that obtained by Steinhardt and Harris.

Curve B of Fig. 1 indicates that the acid-binding capacity of the wool keratin is not affected by formaldehyde treatment. The point of zero combination or isoionic point is in no way shifted owing to formaldehyde fixation.

The data for formaldehyde fixation of wool keratin are of about the same order as those obtaining for collagen. Curve C indicates that at a pH value corresponding to that of zero acid or base binding the formaldehyde fixation curve shows a definite point of inflection. The plateau zone of formaldehyde fixation, pH 6.2 to 9.5, corresponds to a similar zone of base fixation, as shown in Curve A. The writers do not believe that the formaldehyde fixed in this zone corresponds to a reaction with the ε-amino groups of the lysine residues, since these groups are fully regenerated only in a pH range greater than 11.0. The upward sweep of Curve C at pH 11.0 would seem to substantiate this conclusion.

The calculations of Steinhardt and Harris show about 0.044 mM of histidine per gm. of keratin. The increase in fixed formaldehyde between pH 6.1 and 8.0 corresponds approximately to 0.05 mM and therefore the writer believes that the increased fixed formaldehyde at this point corresponds to a reaction between the imidazole group of histidine and the formaldehyde. A similar suggestion has been postulated in a previous study dealing with collagen (5, 6).

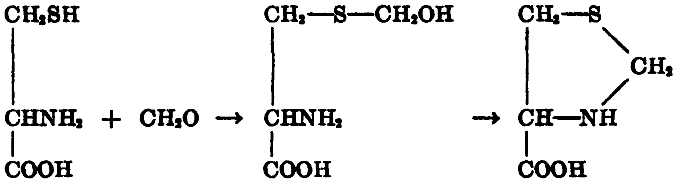
TABLE I

Acidic and Basic Amino Acids in Wool

	per cent	*mM per gm.*
1. Aspartic acid	7.27	0.545
2. Glutamic "	15.27	1.035
3. Amide nitrogen	1.37	0.978
(1) + (2) − (3)		0.602
4. Arginine	10.20	0.586
5. Lysine	3.30	0.226
6. Histidine	0.66	0.044
(4) + (5) + (6)		0.856
(1) + (2) − (3) + (4) + (5) + (6)		1.458
7. Tyrosine	5.80	0.320

The formaldehyde fixed in the pH range 1.0 to 5.0 the writer believes is a reaction between formaldehyde and the acid amide groups, or the imino groups of the polypeptide chain, or possibly both. This same reaction obtains over the entire pH range but is distinctly noticeable in the acid zone. At pH values greater than 6.1, in addition to fixation at the imino or amide groups, increased fixation occurs as the imidazole group of histidine gives up a proton and again at pH 10.5 as the ε-amino groups of lysine are regenerated from their conjugate acid forms through the loss of a proton.

In the specific case of collagen, the formaldehyde fixed between pH 9.5 and 12.0 corresponded to 1 mole of formaldehyde with each ε-amino group of lysine. Such is not the case for the wool keratin in this pH range. A greater amount of formaldehyde is fixed (0.42 mM) between pH 9.5 and 12.0 than the lysine content (0.23 mM) of the wool keratin would account for. This is undoubtedly due to a reaction of the formaldehyde with the sulfur groups of the keratin as postulated by Ratner and Clarke (2). These investigators have shown that for cysteine derivatives formaldehyde may react as follows:



A reaction of this type might well account for the increased formaldehyde fixation over that obtaining for lysine, in the pH range 9.5 to 12.0.

It appears highly improbable that the guanidino group of arginine reacts with formaldehyde to any extent in the pH range studied, since it only disociates at pH values greater than 12.0.

SUMMARY

The acid- and base-binding capacity of purified wool keratin has been studied by an entirely different method from that generally used in such an investigation. The nature of the curve is similar to the titration curves obtained by other investigators.

The acid- and base-binding capacity of formaldehyde-treated wool keratin has been investigated and shows no change in the acid zone or at the zero combination point.

The formaldehyde fixation by wool keratin is given and is somewhat similar to that obtained for collagen. An interpretation of the data is given.

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A CONVENIENT METHOD FOR THE PREPARATION OF SYNTHETIC XANTHOPTERIN*

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(Received for publication, March 13, 1944)

It is now well established that xanthopterin is a hematopoietic substance (1–4) probably closely related to vitamin M (3–6), folic acid (7–10), and vitamin B_c (11). The physiological importance of such a substance makes a practical method for its synthesis of considerable interest.

At the present time there are three methods available for the synthesis of xanthopterin but all suffer from serious disadvantages. The original Purrmann synthesis (12) is claimed by the author to give a yield of 6 per cent in two steps. While such yields have occasionally been obtained in this laboratory, the usual yield is only 2 or 3 per cent. Purrmann has also published a second method (13) but in this one, as in that of Koschara (14), starting materials are used which are not readily available.

Wieland and coworkers (15) stated that leucopterin, which differs from xanthopterin only in having an additional oxygen atom, was unaffected by reducing agents. With the synthetic compound (16) it has been found in this laboratory that leucopterin is readily reduced by sodium amalgam to form xanthopterin and dihydroxanthopterin in excellent yield. Since dihydroxanthopterin is always formed under the conditions that have been investigated, it was found more convenient to use an excess of reducing agent, so that dihydroxanthopterin is the chief end-product. The latter compound may be converted to xanthopterin with little loss by oxidation with silver nitrate in alkaline solution. Purrmann has previously reported the synthesis, by another method, of dihydroxanthopterin and its conversion to xanthopterin by catalytic oxidation (13).

EXPERIMENTAL

Leucopterin was prepared for the reduction by heating 6.3 gm. of 2,4,5-triamino-6-hydroxypyrimidine bisulfite (4, 17) with 25 gm. of oxalic acid dihydrate to 160–170° for 2 hours. After cooling, the crude substance was dissolved by warming with 90 to 100 cc. of 3 N KOH and the resulting solution was poured slowly into 150 cc. of boiling 4 N HCl. After the solution had boiled for a few minutes, the leucopterin was centrifuged in

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* Research paper No. 538, Journal Series, University of Arkansas. This work was supported in part by a grant-in-aid from the Nutrition Foundation, Inc., New York.

100 cc. centrifuge tubes and washed three times with water by centrifugation. The precipitate was all transferred to one tube for the final washing.

After centrifugation excess water was drained from the leucopterin and 20 gm. of 2 per cent sodium amalgam added to the semisolid precipitate in the centrifuge tube. After the reaction mixture cooled, further 20 to 30 gm. portions of sodium amalgam were added at intervals of about 5 minutes until 120 gm. had been used. The mixture was shaken during the reaction and for 10 minutes additionally, or until the tube cooled. The crystalline sodium of salt of dihydroxanthopterin began to separate almost at once. This suspension was then used either for the isolation of dihydroxanthopterin or for the preparation of xanthopterin.

Xanthopterin—The suspension of sodium dihydroxanthopterin was separated from the mercury and the mercury washed with water. The washings were added to the suspension and the latter diluted to about 150 cc. Upon being warmed, the suspended material went into solution. To this warm solution (50–60°) 100 cc. of water containing 15 gm. of silver nitrate were added slowly with vigorous shaking. After standing for 1 hour at room temperature the voluminous black precipitate containing the xanthopterin was centrifuged off and washed once with water. The xanthopterin was extracted by treatment of the precipitate with 240 cc. of 1 N HCl, centrifugation, and reextraction of the residue with two additional 80 cc. portions of 1 N HCl. The combined supernatants were saturated with sodium acetate and the xanthopterin separated out in its characteristic lemon-yellow amorphous form. After being centrifuged and washed twice the compound was redissolved and reprecipitated as before, and then washed four times in the centrifuge. Air-dry yield 1.6 gm., 31 per cent of the theoretical based on the 2,4,5-triamino-6-hydroxypyrimidine bisulfite used. The yield, based on the leucopterin, is above 50 per cent.

The compound prepared thus is identical with xanthopterin prepared by Purrmann's method, as shown by its absorption spectra[1] (Fig. 1) and by its fluorescence characteristics. The biological activity of xanthopterin prepared from leucopterin was determined by feeding it to a vitamin M-deficient monkey and by incubating it with fresh rat liver by the method of Wright and Welch (8). Both tests showed it to be as active as xanthopterin prepared by Purrmann's method.

Dihydroxanthopterin—For the preparation of dihydroxanthopterin the suspension of its sodium salt as obtained above was diluted to 150 cc. and heated until most of the material dissolved. A slight residue was removed by filtration. The clear yellow filtrate was acidified with hydrochloric acid and, after being cooled, the semicrystalline precipitate was removed by centrifugation. After thorough washing in the centrifuge the crude dihydroxanthopterin was recrystallized from boiling 10 per cent sodium carbonate solution. After being cooled, filtered, and thoroughly washed the material was dried at 110° for microanalysis.[3] Yield, 1.6 gm. Corrected for a small amount of ash, the results were as follows:

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[1] The author is indebted to Joseph L. Ciminera of Sharp and Dohme, Inc., for the spectrophotometric analyses.

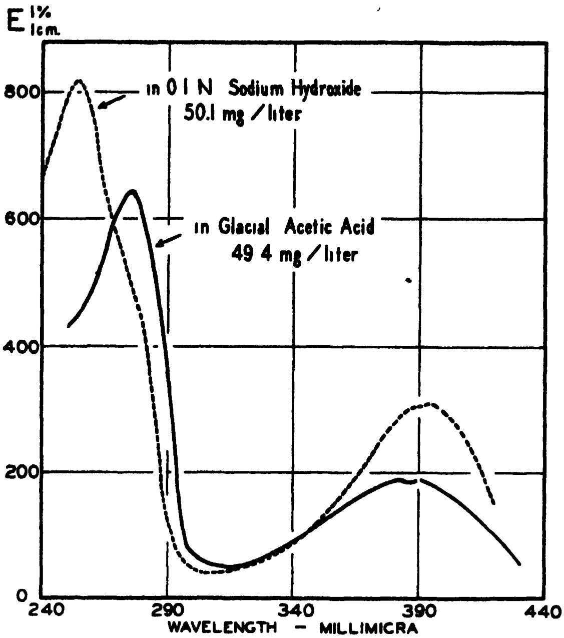

FIG. 1. Absorption spectra of xanthopterin prepared from synthetic leucopterin

$C_6H_7O_2N_5$. Calculated. C 39.77, H 3.90, N 38.67
Found. " 39.07, " 3.93, " 37.16

Dihydroxanthopterin crystallizes from hot sodium carbonate solution or from hot dilute hydrochloric acid in spherical aggregates of colorless well formed acicular crystals, which darken at 285° but do not melt under 295°. The substance is practically insoluble in neutral solutions but readily soluble in alkalies. The sodium salt is sparingly soluble in cold 5 per cent sodium hydroxide, from which it separates in elongated prismatic monoclinic needles with parallel extinction; $\beta = 55°4'$.

Alkaline solutions of dihydroxanthopterin absorb oxygen from the air and rapidly reduce sodium hypobromite, alkaline silver solutions, chloramine-T, quinone, 2,6-dichlorophenol indophenol, and methylene blue.

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[3] Microanalysis by Dr. Carl Tiedcke, New York.

SUMMARY

Xanthopterin and dihydroxanthopterin have been prepared in good yield by the reduction of leucopterin.

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THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDS

IV. THE ACTION OF CHOLINE AND CHOLINE PRECURSORS IN WEANLING RATS*

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(Received for publication, March 3, 1944)

In a previous paper (1) administration of choline to weanling rats was reported to prevent both the fatty infiltration and the low values of liver lecithins which are found in animals on an experimental diet. Investigations in which compounds labeled with isotopes were employed have indicated that methionine (2), ethanolamine (3), serine (4), and glycine (3) may be converted into choline. Accordingly, in the present study, it was thought worth while to determine whether any of these substances could substitute for choline in its effects on the liver lipids of weanling rats. In order to insure an adequate supply of both "methyl donor" and "methyl acceptor" (5), the combination of methionine with each of ethanolamine, serine, and glycine was also tested. Moreover, as high levels of fat in the diet have been shown to increase the amount and the rate of formation of phospholipids in the liver (6, 7), similar experiments have also been done in which the fat content of the diet was increased considerably.

EXPERIMENTAL

Two experimental diets were employed, a low fat diet previously described (Diet 1 (8)) and a high fat diet (Diet 5) containing casein (Labco, vitamin-free) 10 parts, Crisco 25, cod liver oil 5, dextrin 27, sucrose 27, and otherwise identical with Diet 1. The supplements[1] (choline hydrochloride, ethanolamine, *dl*-serine, *dl*-methionine, glycine) were mixed in the diet. Since the daily food consumption was recorded, it has been possible to calculate the actual amount of supplement ingested.

Weanling rats (25 to 35 gm.) were transferred from the nursing mother to either the supplemented or the unsupplemented diets. In most of the experiments, supplementation of the diet for 11 days was initiated immediately; in others a 7 day period was permitted to elapse beforehand.[2] After

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Merck and Company, Inc., Rahway, New Jersey.

[2] Immediate supplementation was the procedure used in our previous experiments on weanling rats. However, the present experiments, in which supplementation is

the experimental period was over, the animals were decapitated. The lipids were analyzed as previously described (9), except that in most cases the determinations were made on the pooled livers of two or more rats. At least two analyses for each experiment were made. Control groups included rats maintained for corresponding periods on the stock diet and on the unsupplemented experimental diets. The analytical values[3] are presented in Tables I and II.

Results

Experiments with Immediate Supplementation—From Table I it is clear that the livers of rats on both Diets 1 and 5 exhibited values for total and choline-containing phospholipids which were markedly low as compared to results on control animals on the stock diet. Supplementation of these experimental diets with choline did prevent to a considerable degree the low values of lecithins. On the contrary, non-choline phospholipids were significantly lowered, resulting in a higher percentage of choline-containing phospholipid in the total phospholipids. These results completely confirm our original observations (1).

Among the experiments with individual supplements (other than choline), only in two experiments (Nos. 8 and 21) out of seven (Experiments 8 to 11 and 19 to 21) did the lecithins appear appreciably higher. As for supplementation with combinations (Experiments 12 to 14 and 22 to 24), a rise in the lecithin level was noticed in Experiments 14 and 23 only. With both combinations and individual supplements, other than choline, the level of non-choline phospholipids remained more or less normal. The percentage of choline-containing phospholipid in the total phospholipids here was uniformly low.

Values for total lipids and neutral fat were higher in the animals on the high fat diet than in those on the low fat diet. It is clear that of the sub-

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initiated after 7 days on the experimental diet, are more strictly comparable with those on more mature rats. In this presupplementation period, it is conceivable that the tissue reserves of various dietary factors may undergo a considerable depletion. This would be in line with the finding of marked changes in the composition of liver phospholipids after only 7 days (8), if actually this composition is controlled by some specific dietary essential. The administration of this substance after the depletion period should result in a return of normal phospholipid values. Accordingly, in our opinion, even a partial restoration of the normal levels following delayed supplementation would be of greater significance than the simple prevention of the changes by immediate supplementation.

[3] In the present experiments, the weight of the dry, lipid-free tissue has been determined and the lipid values calculated also on that basis. As identical conclusions can be drawn from the data, referred to either moist or dry lipid-free tissue, the former method of presenting the results has been retained here.

stances added to each diet only choline prevented the fat infiltration uniformly and completely. However, in three out of the four experiments with ethanolamine or the ethanolamine-methionine combination, the amounts of non-phospholipid fatty acids were distinctly lower than in the

TABLE I

Action of Choline Precursors on Liver Lipids of Weanling Rats (Immediate Supplementation; Values for 1 Gm. of Lipid-Free Tissue)

The supplementation was continued for 11 days.

Experiment No.	No. of rats*	Supplements†	Average daily food intake	Body weight		Liver weight	Total lipids	Phospholipids				Non-phospholipid fatty acids	Unsaponifiable matter
				Initial	Final			Total	Choline-containing		Non-choline-containing		
Stock diet													
			gm.	gm.	gm.	gm.	mg.	mg.	mg.‡	per cent total phospholipids	mg.	mg.	mg.
1– 3	6	None	5.2	29.5	46.8	2.04	44.4	33.5	19.9	59.4	13.6	6.9	3.3
Diet 1 (low fat)													
4– 5	9	None	3.8	27.0	36.8	1.76	107.9	23.4	11.3	48.3	12.1	69.7	7.8
6– 7	10	Choline HCl (35 mg.)	4.3	27.0	34.5	1.44	38.5	24.1	16.2	67.2	7.9	9.0	4.5
8	4	Ethanolamine (13 mg.)	3.8	27.9	36.9	1.61	76.0	27.8	14.0	50.4	13.8	36.4	8.2
9	4	Serine (26 mg.)	4.5	25.6	37.2	1.75	116.8	22.8	12.3	53.9	10.5	75.4	11.1
10–11	10	Methionine (33 mg.)	2.3	28.6	33.0	2.03	140.1	20.6	8.7	42.2	11.9	102.4	6.9
12	6	Methionine (33 mg.), ethanolamine (11 mg.)	1.8	26.5	23.6	1.23	37.0	26.0	11.5	44.2	14.5	7.5	2.7
13	6	Methionine (30 mg.), serine (28 mg.)	2.4	26.3	29.8	1.78	183.6	21.6	9.1	42.1	12.5	138.1	10.1
14	6	Methionine (31 mg.), glycine (25 mg.)	2.4	25.7	29.5	1.60	171.0	25.1	13.0	51.8	12.1	126.6	6.6
Diet 5 (high fat)													
15–16	10	None	2.6	30.7	34.6	1.76	164.0	22.1	9.7	43.9	12.4	122.0	7.7
17–18	11	Choline HCl (25 mg.)	2.9	32.2	36.9	1.58	46.1	25.1	18.7	74.5	6.4	15.2	4.3

TABLE I—*Concluded*

Experiment No.	No. of rats*	Supplements†	Average daily food intake	Body weight		Liver weight	Total lipids	Phospholipids				Non-phospholipid fatty acids	Unsaponifiable matter
				Initial	Final			Total	Choline-containing		Non-choline-containing		
		Diet 5 (high fat)—*concluded*											
			gm.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*‡	*per cent total phospholipids*	*mg.*	*mg.*	*mg.*
19	5	Ethanolamine (25 mg.)	3.3	31.5	37.9	2.13	217.6	25.7	11.8	45.9	13.9	158.0	18.1
20	5	Serine (52 mg.)	4.7	35.2	42.1	2.52	257.4	25.2	10.6	42.1	14.6	199.0	13.3
21	6	Methionine (28 mg.)	1.8	28.5	29.8	1.51	136.0	26.2	12.9	49.2	13.3	95.8	4.4
22	6	Methionine (24 mg.), ethanolamine (19 mg.)	2.2	28.7	33.3	2.07	86.3	23.3	9.5	40.8	13.8	53.3	4.4
23	5	Methionine (24 mg.), serine (24 mg.)	2.4	28.4	39.6	2.36	227.3	21.6	12.7	58.8	8.9	179.5	8.2
24	6	Methionine (25 mg.), glycine (25 mg.)	2.3	30.4	37.5	2.13	219.3	23.2	10.2	44.0	13.0	171.5	7.4

* Kidneys markedly hemorrhagic, Experiment 12 (six kidneys); slightly hemorrhagic, Experiment 22 (six kidneys) and Experiment 21 (one kidney). In none of the other rats were macroscopic hemorrhages visible in the kidneys.

† The figures in parentheses indicate the average daily intake of the supplements.

‡ The range of the values for choline phospholipids, obtained in those groups of experiments in which at least four separate analyses were made, is reported here· Experiments 1 to 3, 17.1 to 21.0 mg.; Experiments 4 and 5, 10.2 to 14.6 mg.; Experiments 6 and 7, 15.3 to 18.5 mg.; Experiments 10 and 11, 8.1 to 10.2 mg.; Experiments 15 and 16, 9.6 to 9.7 mg.; Experiments 17 and 18, 18.3 to 19.2 mg.

controls. In one of these experiments (No. 12) a level of neutral fat as low as in the choline-supplemented groups was observed.[4] With methionine alone or in combination with serine or glycine, the values for non-phospholipid fatty acids were as high or even higher than in the controls.

Larger amounts of unsaponifiable matter were often associated with the presence of an extensive fat infiltration.

Experiments with Supplementation after 7 Days on Experimental Diet—

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[4] The rats in this group failed to grow. This fact should be noted in view of a possible relationship between growth and fat infiltration of the liver (10).

Here (Table II) low lecithin concentrations were again found in the groups (Experiments 26, *a* and 27, *a*) on the unsupplemented diet. Experiments 26, *a* and 26, *b* were run simultaneously, as were experiments 27, *a* and 27, *b*, 3 months later. This time interval may account for the variability of the data. However, in both experiments, choline administration resulted in values for lecithins distinctly higher than in the corresponding controls. Here, too, low values were found for non-choline phospholipids, resulting in a higher percentage of choline-containing phospholipid in the total phospholipids. The lipotropic action of choline is clearly shown in both experiments.

Table II

Effect of Choline Supplementation after 7 Days on Unsupplemented Diet (Values for 1 Gm. of Lipid-Free Tissue)

The supplementation was continued for 12 days.

Experiment No.	No. of rats	Supplements*	Average daily food intake	Body weight		Liver weight	Total lipids	Phospholipids				Non-phospholipid fatty acids	Unsaponifiable matter
				Initial	Final			Total	Choline-containing		Non-choline-containing		
			gm.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent total phospholipids*	*mg.*	*mg.*	*mg.*
		Stock diet											
25	6	None		30.1	59.2	2.49	38.2	34.5	20.0	58.0	14.5	1.6	2.1
		Diet 1 (low fat)											
26, *a*	4	None	4.0	28.6	42.5	2.15	106.2	20.5	8.9	43.4	11.6	71.2	7.4
26, *b*	5	Choline HCl (43 mg.)	4.1	30.1	46.2	2.27	45.5	20.4	13.1	64.2	7.3	18.3	5.0
27, *a*	6	None	3.4	34.1	46.2	2.16	147.7	27.2	14.1	51.8	13.1	101.1	9.3
27, *b*	6	Choline HCl (40 mg.)	3.2	33.9	39.8	2.10	42.0	23.6	17.1	72.5	6.5	13.3	3.8

* The figures in parentheses indicate the average daily intake of the supplement by each rat.

DISCUSSION

The following picture of the liver lipids was uniformly found in the weanling rats on the two experimental diets when these were supplemented with choline: a greater concentration of lecithins, a lower level of non-choline phospholipids, a higher percentage of choline-containing phospholipid in the total phospholipids, and much smaller amounts of neutral fats. With none of the individual substances suggested as choline precursors which we have tested, could this complete picture be duplicated. This was true in general also for the experiments in which additional amounts of the methyl donor and methyl acceptor were supplied.

However, a relatively high lecithin level was found in Experiment 8, in which ethanolamine alone was given. In this experiment, the percentage of choline-containing phospholipid in the total phospholipids was not increased, a finding which was not unexpected, since ethanolamine seems to be able to raise the concentration of non-choline phospholipids (1). In most of the ethanolamine experiments, a more or less definite lipotropic effect was observed. On the whole, therefore, these results may be explained on the basis of a partial conversion of ethanolamine to choline.

In the experiments with methionine,[5] serine, and glycine, no lipotropic action was observed, and the changes in lecithins were of such a small magnitude that their significance must remain doubtful.

In Paper V of this series an interpretation is offered for the differences in the results of the experiments in which choline supplementation was begun immediately or after 7 days.

In conclusion, the results obtained here illustrate a limitation in the physiological significance of data collected through the use of the isotope techniques. In these latter studies, a relatively high proportion of choline appears to be formed from some of its precursors (2–4). However, none of them, in the conditions of our experiments, was capable of substituting effectively for choline in its action on the liver lipids of weanling rats.

SUMMARY

When weanling rats are placed on an experimental diet, values for lecithins are found to be lower than normal and a fat infiltration occurs in the liver. These changes are prevented to a considerable extent by the administration of choline.

The relative ability of various suggested choline precursors to substitute

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[5] Theoretically, in order to supply the same amount of methyl groups, 3 molecules of methionine for 1 of choline hydrochloride are required. Consequently, as in the experiments on choline supplementation 25 to 35 mg. of choline hydrochloride were ingested daily, 75 to 105 mg. of methionine should have been supplied. However, the maximum amounts the rats could ingest without losing their appetite were found to be 25 to 35 mg. of *dl*-methionine daily. These amounts correspond to levels (1.4 to 2.1 per cent, if the methionine content of the diet is included) which are greater than those (0.9 to 1.0 per cent) at which methionine was shown to be lipotropic in weanling rats (11). Investigations now in progress may provide an explanation for this apparent discrepancy.

for choline in these effects has been studied. The substances tested were ethanolamine, *dl*-methionine, *dl*-serine, and glycine and some of their combinations. With none of them could the action of choline be duplicated, although ethanolamine, alone or together with methionine, was effective to a certain extent.

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THE RELATION OF THE DIET TO THE COMPOSITION OF TISSUE PHOSPHOLIPIDS

V. THE ACTION OF CHOLINE, VITAMINS, AMINO ACIDS, AND THEIR COMBINATIONS IN TWO MONTH-OLD RATS*

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(Received for publication, March 3, 1944)

The possible existence of one (or more) dietary factor other than choline, exerting a rôle in the formation of liver lecithins, has been pointed out (1). The present investigation represents an attempt to identify this factor with some known substances whose deficiency in the experimental diet appeared likely.

In previous experiments, the supplementation of the experimental diet with cystine or glycine (amino acids whose content in the casein of the diet is very low) did not restore normal values for liver lecithins in 2 month-old rats. This does not exclude the possibility that the combination of these two substances with choline may be effective.[1]

On the other hand, it may be that one or more of the B vitamins are the missing dietary essential. In this connection, a considerable decrease in the total phospholipids of various tissues, including liver, has been described in pigeons fed polished rice (8) and in rats on so called vitamin B-deficient diets (9). Indeed, there was the likelihood that Diets 1, 2, and 3 (10) contained inadequate amounts of some of the members of the vitamin B complex.[2]

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* Aided by a grant from the John and Mary R. Markle Foundation. A preliminary report was presented before the North Carolina Academy of Science (Forty-second annual meeting, April 30, 1943).

[1] Many examples may be found in the literature which illustrate the effectiveness of the administration of combinations of substances rather than of the individual compounds. Thus, choline and cystine mixtures are more effective than choline alone in protecting rats on synthetic diets against liver necrosis and cirrhosis (2, 3) and in saving the lives of dogs which had received chloroform (4). In chicks on casein diets, various deficiency manifestations are prevented and growth promoted by combinations of glycine and arginine with choline (5) or with cystine (6) or with cystine and chondroitin (7).

[2] The approximate daily intake (calculated from the food consumption) of the B vitamins in the stock diet was (according to the Arcady Farms Milling Company) thiamine 54 to 65 γ, riboflavin 21 to 28 γ, pyridoxine 23 to 47 γ, nicotinic acid 1440 γ, pantothenic acid 200 to 257 γ. The corresponding amounts for the experimental Diets 1, 2, and 3 were thiamine 10 γ, riboflavin 10 γ, pyridoxine 1 γ, nicotinic acid 100 γ, pantothenic acid 1 γ.

Although there is no evidence in the literature of a rôle of vitamins E and K in phospholipid formation, the action of these vitamins has also been tested, as they, too, were probably deficient in our experimental diets.

The present experiments on liver lipids of 2 month-old rats comprise therefore, essentially, a study of the effects of choline, cystine,[3] glycine, and a mixture of pure B vitamins, separately and in combination. A few experiments on various other substances and some of their combinations have also been included.

EXPERIMENTAL

Male albino rats 2 to 3 months old (100 to 120 gm.) were transferred from our stock diet to one of the following experimental diets: Diet 4, containing casein (Labco, vitamin-free) 10 parts, cod liver oil 5, Crisco 5, dextrin 37, sucrose 37, Ruffex 2, salt mixture (Osborne and Mendel (12)) 4; or Diet 6, containing casein 5, dextrin 39.5, and sucrose 39.5, and otherwise identical to Diet 4.

The following substances[4] were tested: choline hydrochloride, glycine, *l*-cystine, *dl*-methionine, lecithin, *dl*-lysine, *dl*-serine, ethanolamine, glutathione, *dl*-glutamic acid, *dl*-tryptophane, *dl*-phenylalanine. Although in most of our former experiments the supplements were given by stomach tube (1), in the present series they were mixed in the diet, except glutathione which was injected daily in isotonic saline.

In most of the experiments, the animals received a daily injection of an isotonic solution (pH 7.4) of B vitamins.[5] The amounts of each supplied daily were as follows: thiamine hydrochloride 50 γ, riboflavin 50 γ, pyridoxine hydrochloride 50 γ, nicotinic acid 1500 γ, calcium pantothenate 200 γ, inositol 200 γ, *p*-aminobenzoic acid 200 γ. In the case of vitamin E, a solution of α-tocopherol[5] in ethyl myristate was emulsified in water in the presence of gelatin and sodium stearate. This emulsion was administered by stomach tube in amounts corresponding to 2 mg. of the vitamin every 2 days. In Experiment 54, an aqueous solution of vitamin K[5]

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[3] Stetten and Grail (11) state that the liver lipids resulting from feeding cystine and choline are "abnormally rich" in lecithins. However, when their data are expressed on the basis of 1 gm. of lipid-free tissue, no increase in choline phospholipids due to cystine becomes evident. The relative significance of results expressed as concentration or referred to the whole liver has been discussed (1).

[4] All these were obtained from Merck and Company, Inc., Rahway, New Jersey, except glutathione and lecithin which were from the Pfanstiehl Chemical Company, Waukegan, Illinois. The lecithin ("pure, from egg"), analyzed by us, contained a considerable amount of acetone-soluble lipids. However, probably only traces of non-choline phospholipids were present, as the ratio of choline to phosphorus in the preparation corresponded to the theoretical value for lecithin.

[5] Generously supplied through the courtesy of Merck and Company, Inc.

(2-methylnaphthoquinone) was added to the vitamin E emulsion, so that each animal received approximately 20 γ every 2 days.

Each experimental group usually consisted of from four to six animals and often more than one group was employed. The animals were decapitated at the end of the period of supplementation. Then the livers were pooled and minced in a meat grinder. At least two samples of the liver mixture were analyzed for liver lipids, as previously described (13). Duplicate analyses for choline, lipoid P, and dry weight[6] were always made.

In the first series of experiments (Table I), administration of the test substances was initiated immediately and continued for 12 days. Here the effects of supplementing the diets with glycine, cystine, methionine, and some of their mixtures, both in the presence and absence of choline and vitamins B, were investigated. In another series (Table II), similar experiments were carried out in which the substances were administered after 7 days on the unsupplemented diet. In addition, the action of vitamins E and K and various compounds, such as ethanolamine, glutathione, and a number of amino acids, was also studied.

Controls included animals maintained on the unsupplemented diets for corresponding periods. Additional analyses for liver lipids of animals on the stock diet have been made, and the results of these and previous (13) determinations have been included in the averages, recorded in Table I. However, for the purpose of a more accurate comparison, values have been obtained for liver lipids of rats which were first maintained on the experimental diets for 7 days and were then replaced on the stock diet for 12 days (Table II).

Results

Phospholipids—In agreement with our previous findings (10), it is apparent from Table I that after rats have been transferred from the stock diet to the experimental diets for 12 days a marked decrease of both total and choline phospholipids occurs, with a lower percentage of the latter in the total phospholipids. A very similar picture was observed after the administration of the B vitamins, B vitamins with each of glycine, cystine, and methionine, or B vitamins with mixtures of glycine plus cystine and glycine plus methionine.

When choline was given (in the free form or combined as lecithin), values for choline phospholipids were appreciably higher than in the controls. On the other hand, levels of non-choline phospholipids were lower, with a

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[6] The conclusions which may be drawn from the data calculated on the basis of the dry, lipid-free tissue are identical with those obtained from the values referred to the moist, lipid-free tissue. For the sake of brevity and uniformity, the latter method of presentation has been retained here.

TABLE I

Effect of Choline, B Vitamins, and Some Amino Acids on Liver Lipids (Immediate Supplementation; Values for 1 Gm. of Lipid-Free Tissue)

The supplementation was continued for 12 days.

Experiment No.	No. of rats	Diet No.	Supplements*	Vitamins supplied	Average daily food intake	Body weight		Liver weight	Total lipids	Phospholipids				Non-phospholipid fatty acids	Unsaponifiable matter
						Initial	Final			Total	Choline-containing		Non-choline-containing		
No supplementation															
					gm.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent total phospholipids*	*mg.*	*mg.*	*mg.*
1- 3	24†	Stock	None				122	5.56	44.5	31.5	19.5	61.9	12.0	8.6	3.6
4	4	6	"	0	8.5	107	95	4.62	110.3	21.3	10.7	50.2	10.6	69.7	12.3
5- 6	10	4, 6	"	B	10.6	107	113	5.55	85.6	21.0	10.6	50.5	10.4	49.3	10.4
Supplements other than choline															
7	5	6	Glycine (44 mg.)	B	9.5	111	108	5.97	72.8	19.1	10.3	53.9	8.8	41.6	7.9
8	5	6	Cystine (50 mg.)	"	10.9	112	120	7.49	175.1	20.6	8.6	41.7	12.0	131.7	9.6
9	5	6	Methionine (141 mg.)	"	10.4	113	124	7.12	126.9	21.2	10.6	50.0	10.6	90.0	6.7
10	5	6	Glycine, cystine (42 mg. each)	"	9.2	116	121	7.08	142.1	21.5	11.2	52.1	10.3	100.3	10.3
11	4	6	Glycine (89 mg.), methionine (134 mg.)	"	9.9	110	120	6.34	125.0	19.6	8.8	44.9	10.8	91.6	4.6

Supplements including choline

12-13	9	4, 6	Choline HCl (41 mg.)	0	9.0	110	114	4.98	36.5	22.0	14.7	66.8	7.3	9.6	3.9
14-15	10	4, 6	Choline HCl (49 mg.)	B	11.7	106	124	5.87	64.0	22.0	15.3	69.5	6.7	31.7	7.1
16	5	6	Lecithin (= 53 mg. choline HCl)	"	9.2	109	103	5.08	41.8	19.5	13.8	70.8	5.7	16.2	4.5
17	4	6	Choline HCl, glycine (49 mg. each)	"	11.1	103	111	5.37	42.3	21.2	15.1	71.2	6.1	15.1	4.5
18	5	6	Choline HCl, cystine (47 mg. each)	0	10.3	103	109	5.55	54.6	21.6	12.7	58.8	8.9	26.6	3.7
19-20	10	6	Choline HCl, cystine (49 mg. each)	B	11.4	109	115	6.49	58.0	22.0	15.4	70.0	6.6	27.2	6.1
21-23‡	15	4, 6	Choline HCl, glycine, cystine (46 mg. each)	0	10.8	108	120	6.19	49.8	23.9	14.9	62.3	9.0	18.6	5.4
24-30‡	35	6	Choline HCl, glycine, cystine (46 mg. each)	B§	10.7	107	115	5.85	53.2	24.2	15.7	64.9	8.5	21.3	5.6
31	3	6	Choline HCl, glycine, methionine (42 mg. each)	"	9.5	110	117	6.30	67.5	19.7	13.2	67.0	6.5	40.4	3.4

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* The figures in parentheses indicate the average daily intake of the supplements by each rat.

† Including individual analyses of ten rats previously reported (13).

‡ The range of the values for choline-containing phospholipids in Experiments 21 to 23 was 14.0 to 15.7 mg. and in Experiments 24 to 30, 14.1 to 17.7 mg.

§ In addition to the solution of pure B vitamins, six rats received also 150 mg. of homogenized pork liver and six other rats 90 mg. of dry yeast daily. One group of four rats received by injection 1.5 mg. of nicotinic acid, and no other B vitamins.

TABLE II

Effect of Choline, Vitamins, and Various Nitrogenous Substances on Liver Lipids (Supplementation after 7 Days; Values for 1 Gm. of Lipid-Free Tissue)

The supplementation was continued for 12 days.

Experiment No	No. of rats	Diet No	Supplements*	Vitamins supplied	Average daily food intake	Body weight		Liver weight	Total lipids	Phospholipids				Non-phospholipid fatty acids	Unsaponifiable matter
						Initial	Final			Total	Choline-containing		Non-choline-containing		
No supplementation															
					gm.	*gm*	*gm.*	*gm*	*mg*	*mg*	*mg.*	*per cent total phospholipids*	*mg.*	*mg.*	*mg.*
32–34	9	Stock†	None			110	164	6.60	65.2	37.8	23.3	61.6	14.5	22.0	3.2
35–36	6	4, 6	"	0	8.0	105	125	6.11	89.3	23.6	12.5	53.0	11.1	54.3	6.0
37–39	11	4, 6	"	B	8.7	104	116	5.67	221.8	22.8	12.3	53.9	10.5	171.6	10.2
40	4	4	"	E	9.2	105	134	7.68	156.6	26.0	15.0	57.7	11.0	113.5	5.7
Supplements including choline															
41	6	6	Choline HCl (44 mg.)	0	9.0	110	105	4.44	57.3	22.5	13.8	61.3	8.7	25.5	6.7
42–43	9	4, 6	Choline HCl (42 mg.)	B	8.3	102	106	4.80	48.3	24.1	13.5	56.0	10.6	18.6	3.7
44–45	10	4	Choline HCl (45 mg.)	E	9.9	106	128	5.47	35.8	22.1	12.8	56.9	9.3	9.5	3.2
46	2	4	Choline HCl (43 mg.), lysine (129 mg.)	B	10.8	122	159	5.87	40.5	26.0	13.6	52.3	12.4	8.6	5.0
47	3	4	Choline HCl, ethanolamine, serine (48 mg. each)	"	13.8	112	150	6.50	33.5	21.0	11.9	56.7	9.1	9.2	3.1

Supplements including choline, glycine, cystine

48	4	4	Choline HCl (48 mg.), glutathione (10 mg.)	B	13.1	101	152	6.56	41.6	24.4	14.5	59.4	9.9	11.8	4.2
49–50‡	8	6	Choline HCl, glycine, cystine, (75 mg. each)	0	10.0	101	103	5.32	43.6	22.1	13.9	62.9	8.2	15.9	4.0
51–53‡	14	4, 6	Choline HCl, glycine, cystine (52 mg. each)	B	9.7	105	109	5.58	74.7	22.8	12.9	56.6	9.9	42.4	5.3
54 *	5	4	Choline HCl, glycine, cystine, (45 mg. each)	B, E, K	12.1	104	148	6.87	40.5	22.2	13.5	64.4	8.7	13.3	3.8
55	4	4	Choline HCl (47 mg.), amino acid mixture§	B	13.5	104	164	8.12	52.6	24.4	13.5	55.3	10.9	21.7	4.3
56	3	4	Choline HCl, glycine, cystine, ethanolamine, serine (48 mg. each)	"	13.8	117	156	7.53	41.9	22.9	14.1	61.6	8.8	14.4	3.2

* The figures in parentheses indicate the average daily intake of the supplements by each rat.

† 7 days on Diet 4 or 6, then 12 days on stock diet.

‡ The range of the values for choline-containing phospholipids in Experiments 49 to 50 was 13.0 to 15.1 mg. and in Experiments 51 to 53, 12.2 to 14.5 mg.

§ The average daily intake of amino acids by each rat was glycine 47 mg., *l*-cystine 47 mg., *dl*-lysine 141 mg., *dl*-tryptophane 9.4 mg., *dl*-glutamic acid 188 mg., *dl*-phenylalanine 28 mg.

consequent increase in the ratio of choline phospholipids to the total phospholipids. If, in addition to choline, there was given glycine or cystine or their combination, or a glycine-methionine mixture, the results in general were very much the same. The presence of B vitamins appeared to enhance to a small extent the amount of lecithins. The highest values for all the phospholipid fractions (approaching the lower range of the normal levels) were found in some of the experiments in which choline, glycine, cystine, and B vitamins were administered simultaneously.

TABLE III

Statistical Study of Changes in Liver Phospholipids

Experimental groups	No of rats and analyses*	Degrees of freedom (n)	Total phospholipids		Choline-containing phospholipids		Non-choline-containing phospholipids	
			mg.	*t*†	*mg.*	*t*†	*mg.*	*t*†
Immediate supplementation								
No supplements or none other than choline (Experiments 4–11)	38 (19)	37	20.7	1.33	10.2	10.99	10.5	10.16
Supplements including choline (Experiments 12–20)	46 (20)	38	21.3	5.05	14.6	2.08	6.7	4.87
Supplements including choline, glycine, cystine (Experiment 21–31)	50 (20)	34	24.2	8.87	15.5	6.68	8.7	6.34
Stock diet (Experiments 1–3)	24 (16)		31.5		19.5		12.0	
Supplementation after 7 days								
No supplements (Experiments 35–40)	21 (14)	28	23.6	1.20	12.9	0.05	10.7	1.03
Supplements including choline (Experiments 41–47)	30 (16)	32	22.7	0.36	13.2	1.36	9.5	0.35
Supplements including choline, glycine, cystine (Experiments 48–56)	38 (18)	22	22.9	14.04	13.8	14.10	9.1	6.28
Stock diet (Experiments 32–34)	9 (6)		37.8		23.3		14.5	

* The figures in parentheses indicate the number of analyses.

† t, according to Fisher (14). The values of t corresponding to a probability of a chance occurrence of 5 in 100 are approximately 2.07, 2.05, and 2.03 when n has the values of 22, 28, and 35 respectively. The corresponding t values for a probability of 1 in 100 are 2.82, 2.76, and 2.72.

From Table II, it is clear that when the animals, after a week on the experimental diets, are placed on the stock diet for 12 days (Experiments 32 to 34), the phospholipid values are completely restored to normal. However, when the animals were maintained for a corresponding period on the unsupplemented diet, as before (10), all phospholipid fractions were below normal. The administration of choline, alone or with various other substances, did not alter this picture substantially, although somewhat higher values for lecithins were occasionally found. Likewise, no definite effects can be ascribed with certainty to vitamins B, E, and K, even when these are given simultaneously (Experiment 54).

Neutral Fats—From Tables I and II, it is clear that more or less high values for non-phospholipid fatty acids result in the groups not receiving choline. In all the other groups, in which choline was supplied, neutral fat values were distinctly lower, as was to be expected.

Statistical Treatment of Data (14)—The effects of vitamin administration on the phospholipid levels were found not to be significant. Accordingly, in order to evaluate statistically the action of choline and other substances, we felt justified in arranging the groups irrespective of the presence of vitamins, as in Table III.

Such a treatment of the experimental results here is arbitrary to a certain extent. However, from Table III it appears that when choline supplementation is begun immediately there are significantly higher concentrations of liver lecithins and lower values for non-choline phospholipids. When cystine and glycine are also given, while there is possibly a further elevation of lecithins, the values for total and non-choline phospholipids do become significantly higher than in the groups receiving choline only. Even in this condition, the concentrations of all the phospholipid fractions remain definitely lower than in animals on the stock diet. On the other hand, in the experiments in which supplementation was initiated after 7 days, no significant effect by the supplements is apparent. Only the differences between the average phospholipid values in the groups on the experimental and stock diets are statistically significant.

DISCUSSION

The present experiments confirm our previous finding that in more mature rats the changes in liver lipids induced by experimental diets cannot be reversed by the administration of choline after 7 days. This is true also when the other known nitrogenous components of the phospholipids are given simultaneously (Experiments 47 and 56).

On the other hand, when choline supplementation is initiated immediately, definite effects on liver phospholipids can be demonstrated in 2 month-old rats, as in weanling rats (1, 15).[7] Still, the existence of an age difference remains probable when the results of the experiments in which choline supplementation was begun immediately or after 7 days are compared. Thus, choline prevents the low liver lecithin values in weanling rats to a greater extent than in more mature animals, whereas it may partially restore liver lecithins in weanlings and not at all in the older animals.

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[7] It should be emphasized that the effects (increase in lecithins, decrease in non-choline phospholipids with a high percentage of choline phospholipid in the total phospholipids) are similar in the rats of both age groups.

The working hypothesis, previously suggested, may offer an interpretation of our results including the present ones. It was then postulated that, in addition to choline, some other factor may be required for the formation of lecithins at a normal rate, this factor being present in the stock diet and possibly being transmitted to the young by the mother. When the animals are transferred to an experimental diet (deficient in this factor), a progressive depletion of the tissue reserves may occur with time. This may be responsible for the differences observed when choline supplementation was begun immediately or after 7 days on the experimental diet. If it is assumed that weanling rats possess larger reserves of this factor, the more marked effects of choline supplementation in these animals under both experimental conditions may be explained. Of course, the utilization of some dietary choline for purposes other than lecithin formation, having thus a "sparing" action on liver lecithins, may also partly account for the preventive effect of choline in the experiments with immediate supplementation.

As for the chemical nature of this hypothetical factor, the present experiments indicate that it cannot be identified merely with the substances or the combinations of substances we have tested.[8] In this respect, a simple deficiency of the vitamins studied here is clearly not responsible for the changes in liver phospholipids observed by us. It is therefore likely that the decrease in total phospholipids described by others (8, 9) may be due to a dietary deficiency of substances other than these B vitamins.

Moreover, it appears that the phospholipid level in the liver bears no direct relationship to the general state of nutrition of the animals, as low lecithin values were found in animals which exhibited an excellent rate of growth. This is particularly noticeable in Experiment 55, in which the most evident deficiencies in essential amino acids had been corrected. The same point may be made from the results of our previous experiments with the 30 per cent casein diet which also provided a satisfactory rate of growth (10).

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[8] Of course, the possibility cannot be excluded that some of these substances may also exert a rôle in the maintenance of the normal composition of liver phospholipids, although other experimental conditions may be required to demonstrate the rôle more clearly. This may apply to the cystine, glycine, choline combination and even to glutathione or some of the vitamins, if more significance could be attributed to some of our present findings.

dl-Methionine in the amounts ingested by our animals (134 to 144 mg. daily) could not substitute for choline hydrochloride (41 to 49 mg. daily) in its action on either the lecithins or the neutral fat levels in the liver. This finding, which is in agreement with our results on weanling rats, is being further investigated ((15) foot-note 5).

SUMMARY

In rats 2 to 3 months old maintained on an experimental diet, the supplementation after 7 days with choline, either alone or in combination with various substances (vitamins B, E, K, amino acids, and other nitrogenous compounds), did not reverse the changes in liver phospholipids induced by the unsupplemented diet.

On the other hand, when choline supplementation was initiated immediately, values for liver lecithins were found which were significantly higher than in the corresponding controls, although lower than normal. This change was accompanied by a decrease in the non-choline phospholipid fraction. The present findings are compared with those obtained in weanling rats.

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THE ORNITHINE CYCLE IN NEUROSPORA AND ITS GENETIC CONTROL

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(Received for publication, February 15, 1944)

It has been emphasized by Haldane (1) that for studies of intermediary metabolism "the new science of genetics furnishes a very powerful method." Such a method is founded upon the general premises that genes control many of the chemical reactions within an organism, and that gene mutations by blocking a reaction chain at various points may, in effect, resolve a metabolic process into some of its constituent stages. For instance, the genetics of such diseases as alcaptonuria and cystinuria have elucidated certain problems in human metabolic processes (2), and studies in the genetics of plant pigments have increased the knowledge of the biochemistry of anthocyanins (3). But the study of metabolism by way of genetic differences in naturally occurring populations is limited not only by the low rate of mutation but also by the lethal character of most mutations of genes controlling vital functions. By increasing the mutation rate of an organism, through irradiation or otherwise, it is possible to create numbers of genetic blocks at various steps in the syntheses of substances or in other processes of metabolism. The problem of preserving mutations ordinarily lethal has been met by Beadle and Tatum (4) in a general course of procedure developed around work with the ascomycetous mold *Neurospora.* The wild type of this organism is able to carry out all the syntheses essential to its normal growth and reproduction if biotin, inorganic salts, and a suitable source of carbon are available. Strains of *Neurospora* are irradiated with x- or ultraviolet rays on the assumption that mutations will be induced in genes controlling the syntheses of such substances as vitamins and amino acids. Mutant strains of this kind cannot grow on merely inorganic salts, sugar, and biotin, "minimal medium," but can be expected to grow if the product of the blocked synthesis is added to the minimal medium.

From irradiated *Neurospora* there has been isolated in this laboratory a series of mutant strains which require for growth the presence of arginine in the culture medium. A study of the specific biochemical characteristics of members of this group of mutants has made it possible to demonstrate in *Neurospora crassa* an ornithine cycle similar to that proposed by Krebs and Henseleit (5) as occurring in the mammalian liver, and to assign various steps in the cycle to the influence of particular single genes. To our knowledge the ornithine cycle has not previously been demonstrated in plants.

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Methods

By convention the class name of any member of a series of biochemical mutants of *Neurospora* from this laboratory is based upon the name of the essential substance which must be added to the minimal medium before the mutant can grow. Thus, the mutants requiring the addition of arginine to the minimal medium, *i.e.* unable to synthesize arginine, are designated as *arginineless*. Individual mutant strains are identified more specifically by a number.

Unless otherwise stated all growth experiments reported were carried out in 125 ml. Erlenmeyer flasks, each of which contained 20 ml. of liquid medium. The basal medium consisted of Fries' No. 3 solution supplemented with trace elements, biotin, and 2 per cent sucrose (6). Supplements to this basal medium were added in such fashion that constant volume and constant concentration of the components of the minimal medium were maintained in each flask. In general, media were sterilized by autoclaving 10 minutes at 15 pounds pressure. All mutant strains were kept in vigorous growing condition by means of frequent vegetative transfers to fresh culture media containing arginine. At fairly frequent intervals the strains were given tests to confirm the constancy of their biochemical characteristics. When more than one mutant strain was involved in an experiment, cultures of identical age were used. Inoculations were made with 1 drop of a sterile suspension of asexual spores. Experimental cultures were grown at 25° for varying lengths of time. Growth was measured by drying the mycelia and weighing.

Results

Individuality of Mutant Strains by Genetic Tests—Fifteen *arginineless* strains have been isolated from x-ray- or ultraviolet-treated wild type *Neurospora*. Of these, some seem to be recurrent mutations of certain genes, but at least seven are demonstrably different from one another, as shown by heterocaryon tests (7) and by crosses between the mutants. Each of these seven mutant strains has been outcrossed, and the results of the crosses indicate that in these strains the inability to synthesize arginine is inherited as a single gene. Details of the genetic findings will be published in another journal.

Biochemical Relationships of Arginineless Mutants—The *arginineless* strains grow at approximately the rate of the wild type if sufficient arginine is added to their culture media. Of these mutants, the slowest growing in liquid culture is Strain 29997, which after a growth of 3 days on a supplemented medium attains a mycelial weight about 85 per cent of that of the wild type. The quantity of arginine per 20 ml. of medium which allows for the maximal growth of mycelium over a 3 day period varies among the mutants from 0.1 to 0.2 mM. For each of the strains the rate of growth is a function of the amount of arginine present in the medium (Figs. 1 to 3).

Tests with various other amino acids show that ornithine and citrulline also permit growth of certain strains. With respect to their ability to grow on these amino acids, the mutant strains may be divided into three groups: Strains 21502, 27947, 29997, and 34105 can grow on the addition of arginine, ornithine, or citrulline to their media; Strains 33442 and 30300 cannot utilize ornithine but do use arginine or citrulline; Strain 36703 grows only in the presence of arginine (Table I). Twenty-three other amino acids were found to be inactive. Neither is any of the mutants able to grow on asparagine, glutamine, guanidine, allantoin, or creatine.

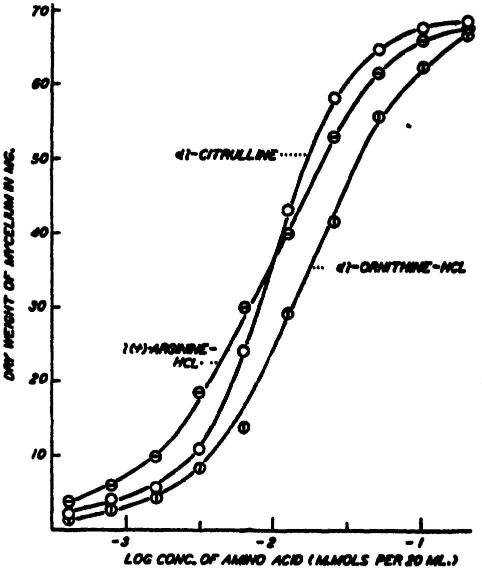


FIG. 1. Growth of Strain 27947 after 3 days at 25° on varying concentrations of arginine, ornithine, and citrulline.

Mutants able to grow on arginine and citrulline but not on ornithine were tested on a mixture of ornithine and urea. Such a mixture does not permit growth when the two substances are autoclaved separately or when the entire medium is sterilized by filtration; growth does occur on media in which ornithine and urea have been autoclaved together. Further analysis

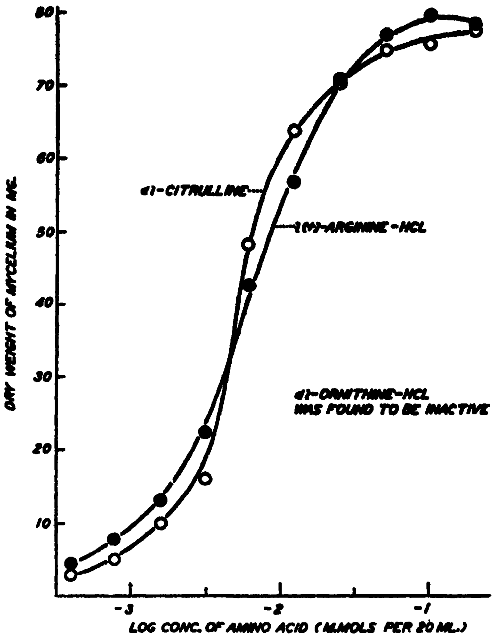


FIG. 2. Growth of Strain 33442 after 3 days at 25° on varying concentrations of arginine, ornithine, and citrulline.

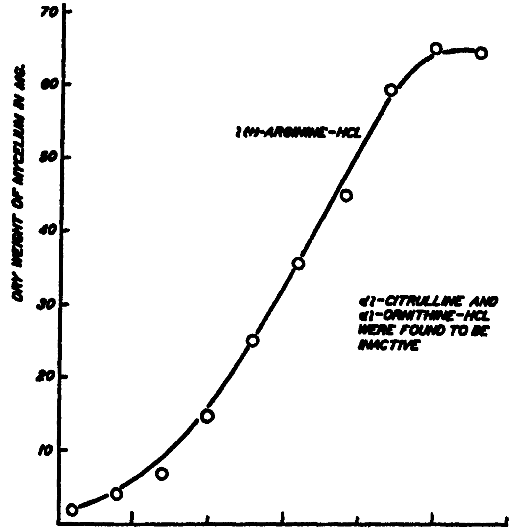


FIG. 3. Growth of Strain 36703 after 3 days at 25° on varying concentrations of arginine, ornithine, and citrulline.

showed, however, that ornithine and urea autoclaved together are converted to citrulline to an extent sufficient to account for the growth of the mutants. Application of Gornall and Hunter's (8) quantitative modification of Fearon's diacetyl monoxime test for citrulline showed that a solution originally containing 0.5 mM of ornithine and 2.5 mM of urea in 10 ml. yielded 0.18 mM of citrulline on being autoclaved at 15 pounds pressure for 15 minutes. A biological assay of the same solution with mutant Strain 33442 gave the same value. The possibility that arginine was also present was eliminated by showing that Strain 36703, which grows on arginine but not citrulline, did not grow on the combination of ornithine and urea autoclaved together.

TABLE I

Growth of Single and Double Mutant Strains

The values represent the dry weight in mg. after 5 days on 0.005 mM of arginine, ornithine, or citrulline.

Mutants	Strain No.	$l(+)$-Arginine HCl	dl-Arginine HNO_3	dl-Citrulline	dl-Ornithine HCl	No supplement to minimal medium
Single	21502	37.2	39.6	37.6	29.2	0.9
	27947	20.9	22.6	18.7	10.5	0.0
	29997	16.7	16.6	15.2	7.7	0.0
	34105	33.2	35.5	30.0	25.5	1.1
	30300	37.6	53.0	34.1	0.8	1.0
	33442	35.0	43.8	42.7	2.5	2.3
	36703	20.4	18.4	0.0	0.0	0.0
Double	27947–29997	17.9		15.5	7.4	0.0
	30300–33442	26.1		32.0	0.0	0.0
	27947–33442	21.8		15.8	0.0	0.0
	21502–33442	25.3		24.5	0.0	0.0
	33442–36703	22.0		0.0	0.0	0.0

In certain experiments it was found that Strain 33442 is able to grow on the minimal medium supplemented with urea. It can be shown, however, that urea as such is not active, and that the effect obtained is due to rise in pH of the medium caused by decomposition of the urea when autoclaved. The pH of Fries' solution alone is about 5.5. Autoclaving 0.5 mM of urea in 20 ml. of solution raises the pH to about 6.7. If flasks of urea medium are sterilized by filtration, the pH does not rise, and Strain 33442 is unable to grow. On the other hand, bringing the minimal medium to pH 6.7 with phosphate-citrate buffer or to pH 8.0 with NaOH permits growth to the same degree as does autoclaved urea. Autoclaved urea medium buffered at pH 5.5 does not support growth. In no case does the minimal medium at high pH values allow for more than 55 per cent of the mycelial weight obtained with an optimal concentration of arginine. Another *Neurospora* mutant, *pyridoxineless*, able to grow on a minimal medium at pH values above that of Fries' solution, is described by Stokes, Foster, and Woodward (9).

The experiments described have shown that not all the mutants can use ornithine or citrulline or both, and that not all those able to use citrulline can use ornithine. But in every case the mutants able to grow on ornithine are able to utilize citrulline as well. This indicates that ornithine and citrulline represent different stages in the course of the biosynthesis of arginine, and that they occur in the order ornithine→ citrulline→ arginine. If ornithine and citrulline were not so related, one might expect to find mutants that use ornithine and arginine but not citrulline. Such mutants have not been found.

The growth requirements of double mutants (Table I), obtained by crossing two different *arginineless* strains, support the above postulated sequence in the biosynthesis of arginine. In each double mutant the growth requirement is determined by the gene whose wild type allele acts nearer arginine in the sequence of the synthesis. Thus in double mutants between a strain which can use all three amino acids and one which can use only arginine and citrulline the requirement is similar to that of the second single strain. A double mutant between Strain 33442, which can grow on arginine or citrulline, and Strain 36703, able to use arginine only, resembles Strain 36703 in its growth requirement. The requirements of double mutants made up of single strains having like requirements are satisfied by the same amino acids utilized by either single strain.

Arginase in Neurospora—Since it could be shown that the synthesis of arginine in *Neurospora* proceeds through a sequence including ornithine and citrulline, it was of interest to determine whether or not the sequence is cyclic in nature. The organism was therefore tested for arginase, by the following procedure: The mold was grown in Fernbach flasks containing 500 ml. of medium. When the mycelial pads had attained maximal growth, they were removed, washed in distilled water, and the excess water was squeezed out through muslin. The pads were weighed, and ground with sand and water in a mortar. The resulting paste was filtered through muslin on a Buchner funnel. Arginase activity was determined in the extract.

Activity of the enzyme was shown by manometric determination (5) of the urea resulting from the incubation of the extract with arginine, and by the isolation and identification of ornithine, as ornithuric acid, from the reaction mixture. Since it was found that extracts of *Neurospora* contain urease as well as arginase, the urea determination gives a minimal measure of the amount of arginine converted.

Table II shows some typical results obtained with three different strains of the *arginineless* series of mutants and with the wild type (Strain 1). The mutant strains were grown in media supplemented with arginine; the wild type was grown in both supplemented and unsupplemented media. The data suggest that the arginase of *Neurospora* may be "partially adaptive;" *i.e.*, although produced by the organism when grown on unsupplemented medium, production of the enzyme is increased when the substrate is added to the medium. It is also shown in Table II that the activity of the *Neurospora* enzyme is increased by manganese, a property shared with arginase of animal origin.

TABLE II

Arginase Activity in Extracts of Neurospora

Concentration of reagents, 1 ml. of extract = 250 mg. of the wet weight of mold, or 63 mg. of the dry weight, arginine hydrochloride 0.238 M, manganese sulfate 0 1 M, glycine buffer, pH 9.5, 1 M. Each flask contained 1 ml. of glycine buffer and water to a final volume of 10.2 ml. After the reagents were mixed in the indicated proportions, toluene was added and the flasks incubated at 34° for 16 to 18 hours. They were then acidified with a few drops of glacial acetic acid, placed in boiling water for 2 to 3 minutes, and filtered. Urea determinations were made on aliquots of the filtrates.

Strain No	Extract	Arginine added	$MnSO_4$ added	Δ urea found	Apparent conversion of arginine
	ml.	*mM*	*ml.*	*mM*	*per cent*
33442	5.0	0.0	0.2	0.000	
	5.0	0.952	0.0	0.163	17.1
	5.0	0.952	0.2	0.435	45.7
29997	6.0	0.0	0.2	0.000	
	6.0	0.476	0.2	0.110	23.1
29738*	5.0	0.0	0.2	0.000	
	5.0	0.476	0.2	0.066	13.9
1†	6.0	0.0	0.2	0.000	
	6.0	0.476	0.2	0.067	14.1
1‡	7.0	0.0	0.2	0.000	
	7.0	0.476	0.2	0.027	5.75

* An *arginineless* strain the same as Strain 30300 biochemically and apparently the same genetically.

† Grown in a medium containing 1 mg. of arginine hydrochloride per ml.

‡ Grown in a medium containing no arginine supplement.

For the isolation of ornithine, 18 gm. (wet weight) of the wild type mold which had been grown on unsupplemented medium were used. The mycelium was ground and extracted as described above. To 62 ml. of the extract were added 0.5 gm. of arginine hydrochloride, 1.4 ml. of 0.1 M $MnSO_4$, and sufficient N NaOH to bring the pH to 9.2. No buffer was used. The mixture was incubated at 34° for 18 hours. The reaction was stopped by acidifying to pH 5.0 with glacial acetic acid; the solution was then placed in boiling water for a few minutes to coagulate the proteins, which were filtered off. An aliquot of the filtrate was removed for urea determination. The analysis showed an apparent decomposition of 20 per cent of the arginine. The remainder of the solution was taken to dryness and extracted with a small volume of alcohol. The residue was taken up in a few cc. of N NaOH and benzoylated by the Schotten-Baumann reaction. 40 mg. of material were obtained which, after recrystallization from 50 per cent alcohol, weighed 20 mg. and melted at 181°; the mixed melting point with authentic ornithuric acid melting at 185° was 183°. Elementary analysis of the sample showed the following composition.

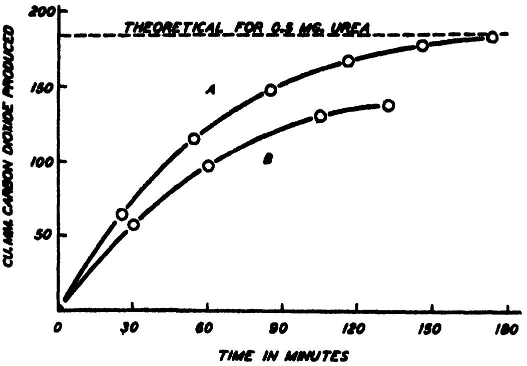


FIG. 4. Urease activity in extracts of the wild type *Neurospora* grown on ordinary medium (Curve A) and on medium containing urea as the sole source of nitrogen (Curve B). 2 ml. of extract per Warburg vessel; 1 ml. is equivalent to 1.4 gm. of wet weight of mold, or 0.35 gm. of the dry weight. Temperature 28.6°.

$C_{19}H_{20}O_4N_2$. Calculated. C 67.03, H 5.93, N 8.23
Found. " 67.05, " 6.03, " 8.11

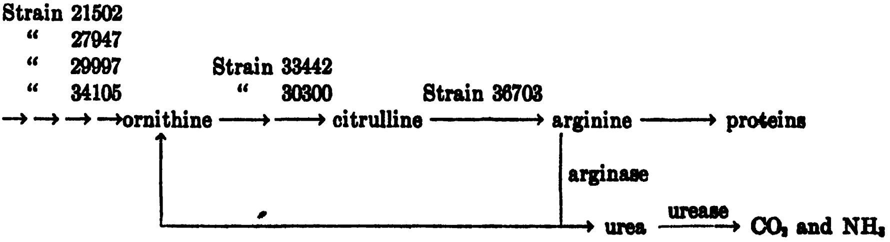
Urease in Neurospora—The subsequent fate of the urea formed in the arginase reaction was found to be cleavage by urease. The mold for experiment was grown in Fernbach flasks, harvested at the end of 7 days, and washed and ground with sand and the minimal amount of water. The resulting paste was centrifuged and the supernatant liquid, containing the enzyme, was diluted with 0.05 volume of 3 N acetate buffer at pH 5.1. 2 ml. of the extract were placed in the main compartment of a Warburg vessel, and 0.5 mg. of urea in 0.2 ml. of water was placed in the side arm. After temperature equilibration the contents were mixed and carbon dioxide production was measured. The results of two experiments with the wild type are plotted in Fig. 4. Control vessels containing boiled extract, or KOH in the alkali well, showed slight or no gas exchange. In a separate experiment it was shown by distillation and titration with standard alkali that ammonia is also a product of the reaction.

Urease activity in *Neurospora* is not increased when the mold is grown on urea as the sole source of nitrogen. There is actually a small decrease (Curve B in Fig. 4). This is probably related to the fact that growth on the urea medium is somewhat abnormal. Measurements of urease activity in the medium on which the mold had grown showed small, barely significant carbon dioxide production. It thus appears that the reaction occurs mainly in the cells.

DISCUSSION

The biosynthesis of a substance like arginine may be expected to proceed as an ordered series of chemical steps. One of the basic concepts derived from the data presented is that genes control in a primary way the single steps making up such a chain of reactions. Mutation involves the loss of ability to carry out a single step in the course of a synthesis, and the mutant genes in the different *arginineless* strains of *Neurospora* are, in effect, stops or blocks at different stages in the biosynthesis. It follows that if the requirements of two different mutant strains are satisfied by the same substance *x* and only one of the strains by a substance *y*, then *y* is a precursor of *x*. Thus arginine, which alone can satisfy the requirements of all the mutants in the series, clearly stands after ornithine and citrulline in the ordered course of the synthesis. Analogous reasoning leads to the conclusion that ornithine is a precursor of citrulline. A similar line of thought has been followed by Tatum, Bonner, and Beadle (10) in establishing the course of synthesis of tryptophane by *Neurospora*.

An integrated scheme of our present interpretation of the known facts concerned with arginine synthesis in *Neurospora* is represented graphically. (Genes controlling various steps in this scheme are identified by the numbers of the mutant strains in which they were first found.)



If, in general, single genes control different primary chemical reactions, and mutations mark interference with particular reactions in an orderly biosynthesis, then the number of different mutations affecting a synthesis may be taken as a minimal measure of the number of steps in the reaction chain. On such a basis, there are no less, and probably more, than seven steps in the synthesis of arginine from sugar and ammonia. Mutations in Strains 30300 and 33442 indicate that the synthesis of citrulline from ornithine is achieved in at least two stages, lending support to the view (11) that the addition of CO_2 and NH_3 to ornithine involves more than one reaction.

This work was supported by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc. The authors wish to express their indebtedness to members of the Genetics Laboratories for the finding and the identification of the mutants described in this paper. The work of producing and isolating some of the mutant strains was supported by a grant from the Research Corporation. The microanalysis of ornithuric acid was carried out by Dr. A. J. Haagen-Smit and Dr. G. Oppenheimer of the California Institute of Technology.

SUMMARY

Seven genetically and biochemically different *arginineless* strains in *Neurospora crassa* are described. In each, the *arginineless* character is inherited as a single gene. The mutant strains are of three general classes: those able to grow on arginine, ornithine, or citrulline; those able to make use of arginine or citrulline but not ornithine; and one mutant with a specific requirement for arginine. This is taken to mean that ornithine and citrulline represent different stages in the synthesis of arginine, the synthesis occurring in the order ornithine→ citrulline→ arginine. Double mutant strains, obtained by crossing different *arginineless* mutants, have growth requirements that confirm the order of synthesis and manner of genetic control postulated above.

Neurospora is shown to have arginase and urease.

The interpretation of the experimental results as a whole is that in *Neurospora crassa* there is operating an ornithine cycle which follows the same general course as proposed by Krebs and Henseleit for urea formation in the mammalian liver. Different steps in the cycle are shown to be governed by the influence of particular single genes.

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THE *d*-AMINO ACID OXIDASE OF NEUROSPORA

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(Received for publication, March 9, 1944)

Among artificially produced mutants of the mold *Neurospora* have been found strains lacking the ability to synthesize specific amino acids (1, 2). In the course of biochemical and genetic studies of this group of mutants it was observed that some of the mutants, *e.g.* those deficient in methionine, leucine, and arginine,[1] are able to utilize racemic mixtures of the amino acids with the same efficiency as the *l*, or physiologically occurring, forms. In the cases of the leucine- and the methionine-requiring mutants it was also possible to show utilization of the α-keto analogues. It thus appeared possible that the mode of conversion of the *d* to the *l* isomers consists in oxidative deamination, followed by resynthesis. A study was therefore undertaken to test the ability of *Neurospora* to oxidize the "unnatural" optical isomers of the amino acids. It was found that extracts of the mold contain a *d*-amino acid oxidase similar in its action to the *d*-amino acid oxidase of mammalian kidney and liver (3). This finding supports the above hypothesis for the conversion of the *d*- to the *l*-amino acids.

Since it appears that the *d*-amino acid oxidase has not been previously described in fungi, a number of experiments were performed on the *Neurospora* enzyme, the results of which are reported here.

Methods

Wild type *Neurospora crassa* was grown in Fernbach flasks containing 500 ml. of the salt-sucrose-biotin medium previously described (4). After 7 to 14 days at 25° the pads were harvested and washed in several changes of the basal salt medium. They were then pressed out through a cloth to remove excess water and weighed. At this stage the pads weighed 4 to 6 gm. each and contained 70 to 75 per cent of water. They were next ground in a mortar, with sand and 2 ml. of M/60 pyrophosphate buffer, pH 8.5, per gm. of wet tissue. The resulting paste was centrifuged at high speed for several minutes, and the supernatant, containing the enzyme, was poured off and diluted with 0.25 volume of 0.25 M pyrophosphate, pH 8.5. The final pH, determined with the glass electrode, was 8.0 to 8.2.

Oxygen consumption was measured in the Warburg apparatus at 28.6°.

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[1] See the papers on the *leucineless* mutant (Regnery, D. C., *J. Biol. Chem.*, **154**, 151 (1944)) and on the *arginineless* mutants (Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154**, 129 (1944)).

2 ml. of the enzyme solution were placed in the main compartment and 0.2 ml. of a M/15 solution of the racemic amino acid in the side arm. In the case of insoluble amino acids, a solution of the sodium salt was used. KOH was placed in the well; the atmosphere was air.

In all experiments the small autorespiration was automatically corrected for by placing enzyme solution in the thermobarometer vessel.

Results

Stoichiometric Relations—In the absence of added substrates the oxygen consumption of the preparation is slight but measurable. On the addition of *dl*-methionine a rapid oxidation was observed. The rate of oxygen consumption remained almost constant until 0.25 mole of oxygen per mole of *dl*-methionine was taken up, and then it rapidly dropped to zero. When *l*-methionine was substituted for the racemic mixture, no oxidation occurred. It is thus evident that the reaction involves the oxidation of *d*-methionine only, with the uptake of 1 atom of oxygen per molecule (Fig. 1). The same relation was found to hold for all other *dl*-amino acids whose oxidation rate was high enough to make an accurate determination of the end-point readily possible; namely, *dl*-phenylalanine, *dl*-norvaline, *dl*-citrulline, *dl*-arginine, *dl*-α-amino-*n*-butyric acid, *dl*-leucine, *dl*-norleucine, *dl*-isoleucine, and *dl*-glutamic acid.

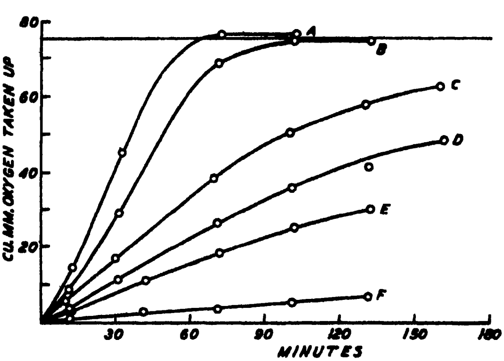


FIG. 1

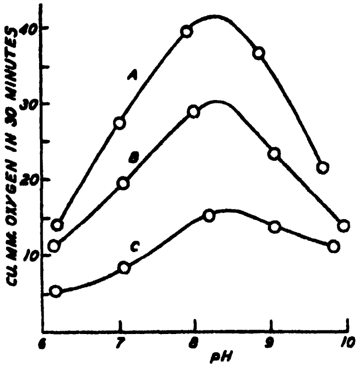


FIG. 2

FIG. 1. Oxidation of some amino acids by *Neurospora* *d*-amino acid oxidase. Curve A, *dl*-methionine; Curve B, *dl*-leucine; Curve C, *dl*-isoleucine; Curve D, *dl*-valine; Curve E, *dl*-lysine; Curve F, *dl*-ornithine. The horizontal line is the theoretical for the uptake of 1 atom of oxygen per molecule of one optical isomer.

FIG. 2. pH curves of *Neurospora* enzyme on *dl*-methionine (Curve A), *dl*-alanine (Curve B), and *dl*-α-amino-*n*-caprylic acid (Curve C).

The keto acid analogue of methionine, α-keto-γ-methiolbutyric acid, was found to be a product of the oxidation of *d*-methionine by the enzyme. It was isolated from the reaction mixture in the form of its 2,4-dinitrophenylhydrazone, melting at 149°, in agreement with the melting point published by Waelsch and Borek (5) and by Cahill and Rudolph (6). When dissolved in alkali the compound gave the red color characteristic of the 2,4-dinitrophenylhydrazones of α-keto acids. Sulfur (by sodium fusion) was present, and sulfhydryl (by the nitroprusside test) was absent.

pH Optimum—The effect of pH changes in the range pH 6 to 10 on the activity of the enzyme was determined. Phosphate buffer was used at pH 6 to 8, pyrophosphate at pH 9 to 10. Determinations were made on three different substrates, *dl*-methionine, *dl*-alanine, and *dl*-α-amino-*n*-caprylic acid, respectively. In all cases a marked optimum at pH 8.0 to 8.5 was observed (Fig. 2).

Effect of Substrate Concentration—The relation between substrate concentration and reaction rate, with *dl*-methionine as substrate, was found to follow the usual hyperbolic law, within experimental limits. The Michaelis constant was approximately 2.5×10^{-4}. This value represents the concentration of *d*-methionine which produces the half maximum velocity, and is equal to the dissociation constant of the enzyme-substrate complex.

Inhibitors—The system is not significantly inhibited by cyanide (0.001 M), iodoacetate (0.001 M), or benzoate (0.01 M). Benzoate has been reported to produce complete inhibition of the kidney *d*-amino acid oxidase at a concentration of 0.01 M (7). On the other hand, drying the tissue with acetone and ether before extracting does not affect the activity of the mammalian enzyme, but in the case of *Neurospora* this treatment results in inactive preparations. The *Neurospora* enzyme is competitively inhibited by isovaline (see below).

Specificity—The enzyme was found to oxidize the *d* forms of most of the amino acids tested. Glycine and *l*-amino acids, with the exception of *l*-glutamate, are not oxidized. *l*-Glutamate is oxidized at less than one-fifth the rate of *d*-glutamate under the conditions of these experiments and presumably by a different enzyme system.

As is the case with the *d*-amino acid oxidase of kidney, *d*-methionine is the substrate most readily attacked by the *Neurospora* enzyme. The oxygen uptake on *dl*-methionine (6.06×10^{-3} M) of sixteen different preparations varied from 64.2 to 148 c.mm. of oxygen per hour per gm. of wet weight of mold, with a mean value of 107 c.mm. The cause of the variability is not definitely known. The experiments have indicated, however, that the variation in activity does not affect the relative rates of oxidation of the amino acids. In the determination of the oxidation rates presented in Table I the activity of each new enzyme preparation was standardized on *dl*-methionine as substrate, to which all other substrates were then referred.

As can be seen from Table I, the following changes in the structure of the substrates destroy their reactivity: shift of the amino group from the α to the β position; replacement of the hydrogen attached to the α-carbon atom by an alkyl group; replacement by methyl groups of both hydrogens attached to the amino nitrogen atom; replacement by methyl groups of the hydrogens attached to the β-carbon atom; substitution of a hydroxyl group on the β-carbon atom; and peptide bond formation through the carboxyl group. The effect of substitutions on the β-carbon atom in lowering the reactivity of the substrate has also been noted in studies of the mammalian *d*-amino acid oxidase (8–10).

Table I

Relative Rates of Oxidation of Amino Acids by d-Amino Acid Oxidase of Neurospora

The mean rate of oxidation of *dl*-methionine = 107 c.mm. of O_2 per hour per gm. of wet mold. All amino acids were tested in a final concentration of 3.03×10^{-3} M in terms of one optical isomer.

Substrate	Relative rate	Substrate	Relative rate
dl-Methionine	100	*dl*-N-Methylleucine	13
dl-Phenylalanine	85	*dl*-α-Aminophenylacetic acid.	About 9
dl-Norvaline	85	*dl*-Tryptophane	" 5
dl-Citrulline	81	*dl*-Ornithine	" 4
dl-Arginine	80	*dl*-Serine	0
dl-α-Amino-*n*-butyric acid.	74	*dl*-Threonine	0
dl-Leucine	66	*dl*-Proline	0
dl-Norleucine	52	β-Alanine	0
dl-Glutamic acid	41	*dl*-β-Amino-*n*-butyric acid	0
dl-Isoleucine	38	*dl*-α-Amino-α-methylbutyric acid	0
d(−)-Alanine	33	*dl*-α-Amino-α-ethylbutyric acid	0
dl-Aspartic acid	29	*dl*-β,β-Dimethyl-α-amino-*n*-butyric acid	0
dl-Alanine	26		
dl-Valine	26	*dl*-N,N-Dimethylleucine	0
dl-α-Amino-*n*-caprylic acid	22	*dl*-Leucylglycine	0
dl-Lysine	14	Glycine	0

Inhibition by Isovaline—A number of the non-reactive amino acids were tested for their effect on the oxidation of methionine. If these substances attach to the enzyme to form an inactive complex, they should competitively inhibit the oxidation of other amino acids. If, on the other hand, no or only slight complex formation occurs, no inhibition is expected. The following compounds were tested: *dl*-serine, *dl*-N,N-dimethylleucine, *dl*-β-amino-*n*-butyric acid, and *dl*-isovaline (α-amino-α-methylbutyric acid). No inhibition of methionine oxidation was found with the first three, even at concentrations which were 10 times higher than the concentration of methionine. It is concluded that in these cases complex formation with the enzyme does not occur.

In the case of the fourth substance tested, isovaline, an inhibition of methionine oxidation was observed. The competitive nature of the inhibition is indicated by its dependence on the concentration of methionine (Table II). The dissociation constant of the enzyme-isovaline complex was calculated by a modification of the equation of Lineweaver and Burk (11),

$$v' = \frac{V(S)K_i}{K_s K_i + K_s(I) + K_i(S)} \tag{1}$$

TABLE II

Inhibition of Neurospora Enzyme by Isovaline

The concentrations of amino acids are given in terms of one optical isomer. The isovaline concentration was 3.0×10^{-2} M in all experiments. A fresh preparation of enzyme was used for each experiment.

Experiment No.	Methionine concentration	p	K_i
	M × 10^3		× 10^3
1	3.0	0.36	4.1
2	3.0	0.36	4.1
	1.5	0.52	4.0
3	1.5	0.44	5.4
	0.75	0.46	8.8
4	3.0	0.36	4.1
	1.5	0.53	3.8
	0.75	0.69	3.4
5	0.75	0.69	3.4
Mean	....	.. . ..	4.6

where v' = the rate of inhibited reaction, V = the maximum rate (proportional to the enzyme concentration), K_i = the dissociation constant of the enzyme-inhibitor complex, K_s = the dissociation constant of the enzyme-substrate complex, (S) = the substrate concentration, and (I) = the inhibitor concentration. In the absence of inhibitor the rate is given by the Michaelis-Menten equation,

$$v = \frac{V(S)}{K_s + (S)} \tag{2}$$

Combining the above equations, one obtains for the inhibited fraction of the rate, p,

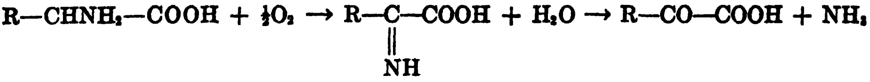
$$p = \frac{v - v'}{v} = \frac{K_s(I)}{K_s K_i + K_s(I) + K_i(S)}$$

from which

$$K_i = \frac{K_s(I)(1-p)}{p(K_s + (S))} \tag{3}$$

Table II shows values of K_i calculated by means of Equation 3, with $K_s = 2.5 \times 10^{-4}$ (see above). The constancy of K_i may be considered good in view of the errors involved in the determination of K_s and of p at low concentrations of substrate.

The failure of isovaline to be oxidized by the enzyme is ascribable to the impossibility of forming the imino structure,



$$\mathrm{R{-}CHNH_2{-}COOH} + \tfrac{1}{2}\mathrm{O_2} \rightarrow \mathrm{R{-}\underset{\underset{\displaystyle NH}{\|}}{C}{-}COOH} + \mathrm{H_2O} \rightarrow \mathrm{R{-}CO{-}COOH} + \mathrm{NH_3}$$

TABLE III

Effect of Chain Length on Reactivity of Straight Chain Amino Acids toward Neurospora Enzyme

Final concentration of amino acids, 3.03×10^{-3} M in terms of one optical isomer. Atmosphere, air; temperature, 28.6°.

Substrate	Oxygen consumed in 15 min.
	c. mm
dl-Alanine	9.4
dl-α-Amino-*n*-butyric acid	17.2
dl-Norvaline	21.4
dl-Norleucine	16.9
dl-α-Amino-*n*-caprylic acid	6.2

In the cases of serine, N,N-dimethylleucine, and β-amino-*n*-butyric acid, imino formation, or its equivalent,[2] is theoretically possible, but the reaction is blocked by factors which prevent attachment of the molecule to the enzyme. In the mammalian *d*-amino acid oxidase, Keilin and Hartree (12) have shown that neither α-methylalanine nor N,N-dimethylalanine is able to form a complex with the enzyme.

Effect of Chain Length—An important relation shown in Table I concerns the effect of chain length on the reactivity of substrates toward *Neurospora* enzyme. With increasing length of the carbon chain in the

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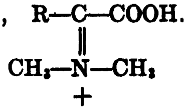
[2] The corresponding oxidation product of N,N-dimethylleucine would be the quaternary ammonium salt,



$$\mathrm{R{-}\underset{\underset{\displaystyle \overset{+}{\mathrm{N}}(CH_3)_2}{\|}}{C}{-}COOH.}$$

homologous series of straight chain, monoaminomonocarboxylic acids, the oxidation rate first rises to a maximum at a length of 5 carbon atoms (norvaline) and then drops off. Since the data in Table I were obtained at different times, with a fresh enzyme preparation each time, it appeared desirable to check this relation on a single preparation. This was done with the results shown in Table III. These data corroborate the previous result.

It seems clear from these findings that an optimum chain length exists among the substrates of the *Neurospora* enzyme. The effect of various substitutions and internal rearrangements on the reactivity of the substrate may thus in part be ascribed to the changes they produce in the length of the molecule. Published reports do not indicate a similar dependence in the case of crude mammalian *d*-amino acid oxidase. In the case of the purified mammalian enzyme, it appears that rate data are not available for a sufficient number of substrates to decide the point.

DISCUSSION

The function of *d*-amino acid oxidase in the metabolism of *Neurospora* is unknown. Any explanation which is based on the hypothesis that the organism may encounter racemic amino acids in nature, or that it may produce them in the course of the digestion and assimilation of proteins, appears unacceptable, since the wild type of *Neurospora* is able to synthesize all of its amino acids from carbohydrates and inorganic nitrogen; it is consequently independent of external supplies of amino acids. If the enzyme serves a useful purpose, it would therefore seem to be concerned with products of the organism's own metabolism. This suggests the possibility of symmetric synthesis of amino acids by the mold. The applicability to *Neurospora* of the recent finding by Shemin and Rittenberg (13) that *d*-glutamic acid and *d*-tyrosine are not synthesized by the riboflavin-deficient rat is an open question.

In amino acid-deficient mutants of *Neurospora*, present evidence suggests that the *d*-amino acid oxidase plays an essential part in the transformation of *d*-amino acids (supplied from the outside in racemic mixtures) to *l*-amino acids. Thus, *d*-methionine, *d*-leucine, and *d*-arginine are all rapidly oxidized by the enzyme and are efficiently utilized by the corresponding mutant strains. In the cases of methionine and leucine the evidence is more complete, in that utilization of the α-keto analogues has also been found. The α-keto analogue of arginine has not been tested. Further evidence, of an indirect kind, comes from the tryptophane-requiring mutants. Tatum and Bonner (14) have shown that tryptophane synthesis in *Neurospora* occurs by a condensation of indole with *l*-serine. *dl*-

Serine is only one-half as effective as *l*-serine in promoting this reaction in experiments *in vivo* (15), indicating that *Neurospora* is unable to convert *d*- to *l*-serine. This finding is in harmony with the observation that *d*-serine is not attacked by the *Neurospora* enzyme. Similar evidence for other amino acids has been obtained with mutants currently under investigation and will be published at a later date.

This work was supported by grants from the Rockefeller Foundation. The author is indebted to Dr. David Bonner for samples of the following amino acids: *dl*-proline, *dl*-N-methylleucine, *dl*-N,N-dimethylleucine, and *dl*-β,β-dimethyl-α-amino-*n*-butyric acid. A sample of *d*(−)-alanine was generously provided by Professor M. S. Dunn of the University of California, Los Angeles.

SUMMARY

1. Extracts of *Neurospora* contain a *d*-amino acid oxidase similar in its action to the *d*-amino acid oxidase of mammalian tissues.

2. The pH optimum of the system lies at pH 8.0 to 8.5.

3. The enzyme is destroyed by drying, but is not inhibited by cyanide, iodoacetate, or benzoate. It is competitively inhibited by isovaline.

4. The *d* forms of the following amino acids are rapidly oxidized: methionine, phenylalanine, norvaline, citrulline, arginine, α-amino-*n*-butyric acid, leucine, norleucine, isoleucine, and glutamic acid. The following are slowly oxidized: aspartic acid, valine, alanine, α-amino-*n*-caprylic acid, lysine, α-aminophenylacetic acid, tryptophane, ornithine, N-methylleucine. The following are not oxidized: glycine, serine, threonine, proline, β-alanine, β-amino-*n*-butyric acid, α-amino-α-ethylbutyric acid, β,β-dimethyl-α-amino-*n*-butyric acid, N,N-dimethylleucine, leucylglycine, and isovaline.

5. The activity of the enzyme shows a marked dependence on the chain length of the substrate. It was found that an optimum chain length exists.

6. The rôle of *d*-amino acid oxidase in the wild type and in mutants of *Neurospora* is discussed.

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A LEUCINELESS MUTANT STRAIN OF NEUROSPORA CRASSA

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(Received for publication, January 21, 1944)

It has been shown by a number of investigators that genes influence specific biochemical reactions (1). Beadle and Tatum (2) have described a method by which gene mutations influencing the production of known substances can be induced and detected in the red bread mold *Neurospora*. This organism requires for growth only inorganic salts, nitrate or ammonium nitrogen, a carbon source, and biotin. Mutant strains have been isolated each of which requires for growth a vitamin, amino acid, or some other substance in addition to the materials present in the basal medium. Several such mutant strains have been found to require the amino acid leucine for normal growth, and these were made available to the writer for detailed study. At the outset it was appreciated that such a *leucineless* mutant might serve as a basis for a microbiological assay for leucine. After extensive tests of three mutant strains, the particular one described in this paper, Strain 33757, was selected as the most suitable. This originated in material treated with ultraviolet light.

On the basis of the results described in this paper Ryan and Brand have used Strain 33757 to determine leucine in protein hydrolysates and have shown the *Neurospora* method to compare favorably with the isotope dilution and solubility product methods. The details of the method as used by Ryan and Brand are given in the following paper (3).

Genetic Analysis—*Neurospora crassa* is heterothallic; *i.e.*, produces ascospores only if strains of the two mating types are put together. When crosses are made between strains of opposite mating type, fertile fruiting bodies are produced which contain asci, each having eight sexual spores (ascospores) arranged in linear order. If a mutant strain such as *leucineless* differs from the wild type by a single gene, it follows from the mechanism of the cell divisions by which the eight ascospores are produced from a single diploid zygote nucleus that each ascus from a cross of the mutant strain with the wild type would contain four spores carrying the mutant allele and four carrying the original normal allele. Thus if the spores are ger-

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* Nutrition Foundation Fellow at Stanford University. The author is indebted to the Nutrition Foundation, Inc., for supporting the fellowship under which the work reported was done. He likewise wishes to express his gratitude to members of the Genetics Laboratories of Stanford University for helpful suggestions. Dr. D. M Bonner kindly supplied the keto acid analogue of leucine as well as leucic acid. Tripeptides were obtained from Dr. M. J. Johnson of the University of Wisconsin.

minated, four of the resulting haploid mycelia should be able to grow on media deficient in leucine and four should be unable to do so. The arrangement of pairs of spores carrying mutant or normal alleles indicates the distance of the segregating gene from the centromere of the chromosome in which it is located (4).

From crosses of *leucineless* (Strain 33757) and wild type strains, spores from 131 asci were isolated, their positions in the asci recorded, and cultures established from them on a medium containing leucine. Transfers of conidia were then made to a medium in which no leucine was present and the cultures classified as to their ability to grow. In all instances the results were consistent with the assumption of a single gene difference between *leucineless* and normal strains. When all eight spores germinated, four of the resulting strains grew in the absence of leucine and four did not. In 110 asci the two types of spores were arranged in groups of four, indicating first division segregation, while in the remaining twenty-one asci the types were in groups of two as expected from second division segregation. This indicates that the gene concerned is approximately eight map units from the centromere.

In genetic crosses *leucineless* segregated independently of sex (mating type) and of the sex-linked character *albino-1* and is therefore not located on the sex chromosome. It does show linkage with a morphological character designated *5801*. After a preliminary cross gave rise to the double mutant strain *leucineless-5801*, this double mutant was crossed to the wild type, and spores from forty-two asci isolated in order. An analysis of the results summarized in Table I indicates that gene *5801* is close to the centromere of the chromosome in which it is located and that the *leucineless* gene is about four map units from *5801*.

Specificity of Amino Acid Requirement of Strain 33757—Subcultures of the original and of the genetically derived strains of Strain 33757 have been maintained at 20–25° on media fortified with leucine and under these conditions all stocks have thus far retained the specific characteristics of the original culture. Of the amino acids investigated only leucine supports growth of this mutant. A negative response was found with each of the following: *dl*-alanine, *l*(+)-arginine, *l*(−)-asparagine, *l*(−)-cystine, *l*(+)-glutamic acid, glycine, *l*(−)-histidine, *l*(−)-hydroxyproline, *dl*-isoleucine, *l*(+)-lysine, *l*(−)-methionine, *dl*-norvaline, *dl*-norleucine, *dl*-phenylalanine, *l*(−)-proline, *dl*-serine, *dl*-threonine, *l*(−)-tryptophane, *l*(−)-tyrosine, and *dl*-valine. The growth-promoting activity of the keto and hydroxy acid analogues of leucine and certain other related compounds is considered in a subsequent section.

Germination of Conidia in Response to Leucine—Germination of conidia (asexual spores) is readily observed in hanging drop cultures. Spores of

Strain 33757 fail to germinate in a medium which contains no leucine or $l(-)$-leucine in a concentration of less than 0.5 γ per ml. At 25° signs of germination are usually apparent in 4 to 5 hours, although this period is markedly influenced by the age of the spores, their degree of desiccation, and their size. Variations in sucrose, biotin, or inorganic salt concentration do not appreciably affect the time required for germination.

From these observations it is clear that germination of conidia can be used to estimate leucine concentrations. This method is particularly useful for bioassays in instances in which only small amounts of material are available.

Quantitative Dependence of Mycelial Growth on Available Leucine—Growth responses in *Neurospora* can be measured in several ways. One of these involves the measurement of rate of progression of a mycelial frontier along the surface of an agar medium (5). This method is unsatisfactory for *leucineless* Strain 33757, because at intermediate leucine concentrations growth is sparse and the mycelial frontier is therefore not well defined. In a second method the dry weight of mycelia produced in liquid medium is measured over a limited time interval; *e.g.*, 3 days (6). As a third procedure growth may be measured as the maximum mycelial dry weight obtained on a given substrate. This "total growth" method has proved most satisfactory for *leucineless*, and all data presented are based on it.

TABLE I

Data on Segregation of Leucineless in Asci from Cross of Normal and Double Mutant Leucineless, 5801

Inability to grow in the absence of leucine is indicated by the symbol *lc*. Mutant *5801* is designated by its number. + indicates the non-mutant conditions; + to the left indicates ability to grow in absence of leucine; + to the right indicates a strain that is not mutant *5801*.

No. of asci	Constitutions of spores			
	Pair 1	Pair 2	Pair 3	Pair 4
20	*lc 5801*	*lc 5801*	+ +	+ +
19	+ +	+ +	*lc 5801*	*lc 5801*
1	*lc 5801*	+ *5801*	*lc* +	+ +
1	*lc* +	+ +	+ *5801*	*lc 5801*
1	+ +	*lc* +	*lc 5801*	+ *5801*

Quantitative measurements of growth were made by culturing the mutant strain in Erlenmeyer flasks containing a liquid basal medium composed of a mixture of inorganic salts, sucrose (2 per cent), and biotin (6). Leucine was added in the form of a methionine-free preparation of $l(-)$-leucine. Recrystallization of this did not change its growth-promoting activity. Inoculations were made with suspensions of asexual spores. Although the size of the inocula was found to be without effect on the final weight, each flask of an experimental series was inoculated with a fixed amount of a single suspension. Flasks of varying sizes were employed, depending on the volume of media. For 25 ml. or more of medium, 250 ml. flasks were used, while for smaller volumes 50 and 125 ml. flasks served. Cultures were incubated at 25° and shaken twice daily to prevent sporulation. Mycelial weights were determined after the mycelia were removed, the excess water squeezed out, and the material dried at 90–100° for 4 hours or more.

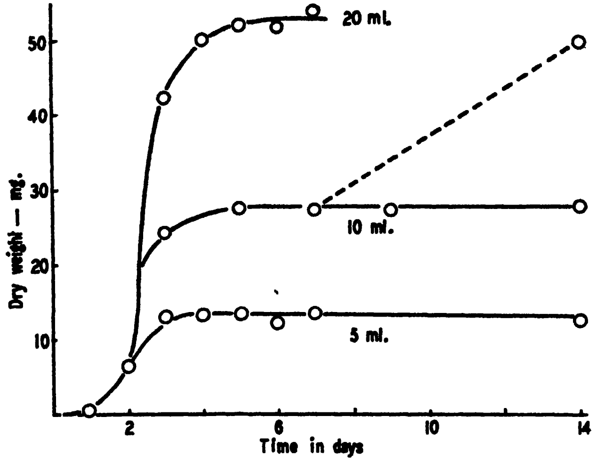

FIG. 1. Dry weight of mycelia produced by Strain 33757 grown for varying times on varying amounts of $l(-)$-leucine. The concentration of leucine is kept constant at 0.1 mg. per ml. and the amount varied by varying the volume of medium as indicated. The dotted line indicates additional dry weight produced on the filtrate of a 7 day 10 ml. culture following the addition of 1 mg. of $l(-)$-leucine and reinoculation. Each point represents the average calculated from two to forty-one determinations.

The stock used for quantitative work was the double mutant *leucineless-albino-1* (Strain 33757-4637). The albino character was added to increase the chance of detecting possible contamination with non-leucineless strains, most of which would have salmon-colored conidia. Stock cultures were maintained on agar slants of the minimal medium fortified with leucine.

The dry weights attained at different times with three volumes of medium are indicated in Fig. 1. It is observed that dry weights reach maximal values and are maintained at a constant level for at least 7 days. That the amount of leucine employed is limiting is shown by the fact that if the mycelium is removed from a culture after cessation of growth, the filtrate autoclaved, leucine added, and the culture reinoculated, further growth is obtained (Fig. 1). Addition of fresh leucine-free medium to a culture in which growth has ceased does not result in additional growth. Furthermore, the filtrate of a culture in which growth of the mutant has ceased will support growth of a wild type strain.

It will be noted in Fig. 1 that the dry weights are proportional to the amount of leucine and in this case to the amount of medium. If the volume

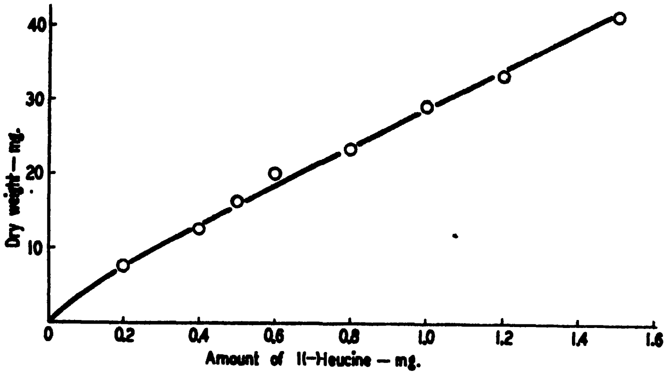

Fig. 2. Relation of dry weight production by Strain 33757 to $l(-)$-leucine concentration. The cultures were grown in 10 ml. of medium in 50 ml. flasks for 7 days at 25°.

Table II

Influence of Amount of Sucrose on Weight of Mycelium Produced on Constant Amount (1 Mg.) of Leucine

The cultures were incubated 9 days.

Sucrose per flask	Volume of medium per flask	Average weight of mycelium
mg.	*ml.*	*mg.*
0.20	10.0	27.6
0.20	20.0	27.5
0.20	50.0	27.2
0.40	10.0	32.9
0.40	20.0	32.8
1.00	50.0	41.1

of medium is kept constant, however, a concentration curve showing a slight deviation from a linear relation is obtained (Fig. 2).

If the absolute amount of sucrose is increased, an increased growth of mycelium is obtained. This holds under conditions of constant concentration or constant volume (Table II). The limited growth at lower sucrose levels is not the result of an insufficient carbon supply, since in all cases the filtrates were able to support further growth when supplemented with leucine.

Utilization of d(+)-Leucine, α-Ketoisocaproic Acid, and Leucic Acid—α-Ketoisocaproic acid, the keto acid analogue of leucine, is as active as *l*(−)-leucine both in initiating and supporting growth of Strain 33757. Since wild type *Neurospora* contains *d*-amino acid oxidase which is active in oxidizing *d*(+)-leucine to its α-keto derivative (7), one might expect the *leucineless* mutant to utilize *d*(+)-leucine along such a pathway. The growth-promoting activities of racemic and natural leucine are compared in Table III. At a concentration of 1 mg. per 10 ml. the activities of *dl*- and *l*(−)-leucine are equal, but at lower concentrations the activity of the *dl* mixture is less. If mycelia are removed from cultures initially containing *dl*-leucine when they have produced approximately half the weight expected at completion of growth, the reautoclaved culture medium will not support growth of the *leucineless* strain. Presumably only *d*(+)-leucine remains in the medium. If *l*(−)-leucine is added to such a culture medium, growth is supported to the extent expected on the assumption that under these conditions both isomers are used. It is possible that this behavior is the result of the *d*-amino acid oxidase activity of the spores being too low to provide enough α-keto acid to initiate growth, but that once mycelial growth is started *d*(+)-leucine can be utilized.

TABLE III

Dry Weight of Mycelia Produced in Cultures Containing dl-Leucine or l(−)-Leucine

In all cases the amount of leucine supplied per flask was 1.0 mg.

Time of incubation	Volume of medium	Dry weight of mycelia on	
		dl-Leucine	*l*(−)-Leucine
days	*ml.*	*mg.*	*mg.*
3	10	24.4	25.1
	20	19.1	
	30	17.4	
7	10	29.0	28.1
	20	24.0	27.6
	30	19.2	27.2
9	10	30.8	29.2
	20	25.4	
	30	23.3	

Leucic acid, the hydroxy acid analogue, will replace leucine, but only after an initial stimulation with the amino acid. If no leucine is present, the growth on leucic acid is restricted to a few hyphae; with the addition of a small amount of leucine (0.02 mg. per 10 ml.) the response to 1 mg. of leucic acid is equal in rate and amount of growth to that observed in a corresponding amount of leucine. The "adaptation" to leucic acid does not carry over through conidial transfers. Variations in pH over the range 3.9 to 8.0 and in temperature from 20-35° did not result in the initiation of growth on the hydroxy acid. Frequently when a very large inoculum is used, growth will occur in media containing leucic acid alone, possibly because an appreciable amount of leucine is included in the inoculum.

Influence of Other Substances—On the supposition that other compounds related to leucine or to the metabolism of leucine might satisfy the growth requirements of Strain 33757, the following substances were tested individually and in the presence of leucine: acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric, *n*-caproic, and isocaproic acids, acetone, isobutyraldehyde, isovaleraldehyde, isoamyl alcohol, and isoamylamine. In the absence of leucine none of these showed activity. When added to media containing leucine, however, isovaleric acid, isovaleraldehyde, and to a lesser extent isoamyl alcohol markedly increased the yield of mycelium. For example, 0.001 ml. of isovaleric acid or isovaleraldehyde and 1 mg. of *l*(−)-leucine in 10 ml. of medium increased the 9 day yield from 28 mg. to 42.5 mg. (Fig. 3). A similar increase was produced by the addition of 0.5 ml. of isoamyl alcohol. Higher concentrations of these compounds were inhibitory. All attempts to obtain growth on isovaleric acid or aldehyde alone by transferring from a medium containing *l*(−)-leucine were negative. In all cases cultures yielding high dry weights were checked and failed to grow on leucine-free medium, showing that neither reversion nor contamination had occurred.

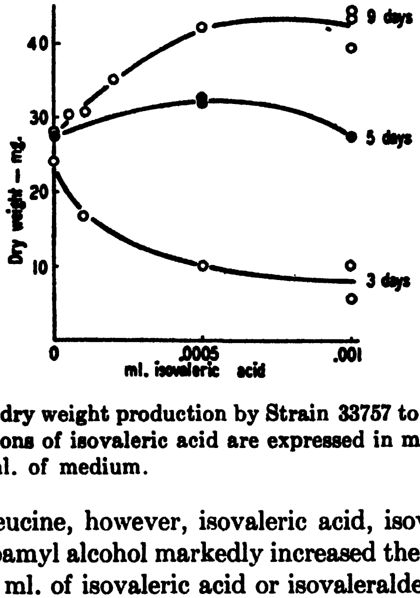

FIG. 3. Relation of dry weight production by Strain 33757 to isovaleric acid added to the media. Additions of isovaleric acid are expressed in ml. of acid per culture flask containing 10 ml. of medium.

Effects of Other Variables—Increasing salt concentration up to twice that ordinarily used has little or no effect on the amount of dry weight produced.

Within a pH range of 3.9 to 7.6, the hydrogen ion concentration of the culture medium has an insignificant effect on the weight-leucine ratio. This is likewise true of temperature variations between 20–30°, but above 35° there is a marked falling off of dry weight. At 40° the mycelial weight per mg. of leucine was 17.6 mg., whereas at 25° the corresponding value was 28 mg. under the conditions reported in Fig. 2.

Hydrolysis of Peptides and Proteins by Strain 33757—The dipeptides, *dl*-leucylglycine and glycyl-*l*-leucine, are utilized by this mutant although in each case growth is initially slower than on leucine. The final weights attained on glycyl-*l*-leucine are equal to those induced by an equimolar quantity of leucine, while those attained with *dl*-leucylglycine are lower than would be expected on the basis of complete hydrolysis and utilization. Leucyldiglycine is apparently not hydrolyzed, since a mixture with leucine gives values no greater than the leucine controls.. Tested in the same fashion, glycylleucylglycine shows slight activity. Since the production of peptidases frequently is increased by growth on unhydrolyzed proteins,

TABLE IV

Leucine Values for Casein As Measured by Neurospora Bioassay*

In all cases, measurements were made at 25° in 50 ml. flasks containing 10 ml. of culture medium.

Treatment of casein	Amount of casein	Amount of *l*(−)-leucine	Incubation period	Dry weight of mycelium	Calculated leucine	Leucine content	Recovery of added leucine
	mg	*mg*	*days*	*mg.*	*mg.*	*per cent*	*per cent*
HCl-hydrolyzed	10	0	8	31.1	1.1	11	
				32.8	1.2	12	
	5	0	8	19.8	0.65	13	
				20.7	0.70	13	
	5	0.5	8	29.7	1.0		91
				30.8	1.1		
	0	0.5	8	15.9			
				16.1			
Tryptic-hydrolyzed	10	0	5	31.6	1.1	11	
			7	31.5	1.1		
	5	0.5	5	30.3	1.05		100
			7	30.2	1.05		
	0	1.0	5	29.8			
			7	28.1			
Unhydrolyzed	10	0	8	29.7	1.05	10.5	
				30.5	1.05	10.5	
				31.1	1.1	11.0	
			10	29.6	1.05	10.5	

* S. M. A. Corporation, vitamin-free preparation.

attempts were made to obtain growth on the three inactive peptides by making use of mycelial-transfers from cultures growing on gelatin, casein, egg albumin, or glycyl-*l*-leucine. In all cases these attempts were unsuccessful.

The *leucineless* mutant can hydrolyze proteins and thereby satisfy its leucine requirement if mycelial inocula are used or if growth is started by adding a small amount of free leucine. Casein and zein produce abundant growth in 5 days. Hemoglobin and gliadin are likewise rather readily broken down, whereas gelatin and egg albumin are more slowly utilized. Table IV summarizes preliminary data on the leucine content of casein. The results obtained with the unhydrolyzed protein, an acid hydrolysate, and a tryptic digest are in reasonable agreement. On the basis of this general procedure Ryan and Brand have developed a quantitative method for the determination of leucine in protein hydrolysates, which is reported in the following paper (3).

DISCUSSION

It is assumed that in Strain 33757 leucine is not synthesized because of the inactivation or loss of a gene which is necessary for some one step in the biosynthesis of leucine. This need not imply that there is only one gene involved in this synthesis. In fact, it seems more likely that there are as many genes concerned as there are steps involved in the synthesis. It might reasonably be expected that precursors entering into the system prior to the reaction blocked in Strain 33757 would be inactive, whereas those that enter reactions subsequent to the blocked reaction would be active in promoting growth. On such a basis it is difficult to determine from the available information what reactions are normally involved in leucine synthesis and which of these is blocked in the *leucineless* strain. The keto acid analogue of leucine is the only compound tried that is the equivalent of leucine for the mutant strain. It is therefore probably a precursor of leucine. Leucic acid may be utilized via oxidation to the keto acid. Since isovaleric acid or aldehyde and isoamyl alcohol are not active in initiating growth of the mutant, it seems likely that they are not normal precursors but that they may be converted into normal precursors or into leucine by reactions that are not part of the normal course of synthesis of leucine in *Neurospora*.

SUMMARY

An ultraviolet light-induced *leucineless* mutant strain of *Neurospora crassa* is reported which is differentiated from normal by a single gene.

The *leucineless* strain responds specifically to leucine and its keto acid analogue. Conidia of this strain fail to germinate and mycelia fail to grow

in the absence of leucine or α-ketoisocaproic acid. Other amino acids and various other compounds related to leucine are inactive in inducing germination or growth. Leucic acid shows growth-promoting activity if growth is initiated by adding small amounts of *l*(−)-leucine or its keto acid analogue. Isovaleric acid, isovaleraldehyde, isoamyl alcohol, and *d*(+)-leucine in the presence of *l*(−)-leucine increase the weight over that of the *l*(−)-leucine controls but by themselves will not initiate growth.

Under standard conditions the dry weight of mycelium produced by *leucineless* in liquid culture is approximately proportional to the leucine available. The weight-leucine relation is stable over rather wide variations in pH, temperature, salt concentration, and presence of various extraneous substances. It is, however, influenced by wide variations in the sucrose-leucine ratio.

A *leucineless* mycelium, once growth is started, is capable of obtaining the leucine it requires for further growth from certain peptides and proteins.

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A METHOD FOR THE DETERMINATION OF LEUCINE IN PROTEIN HYDROLYSATES AND IN FOODSTUFFS BY THE USE OF A NEUROSPORA MUTANT*

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(Received for publication, March 20, 1944)

The first amino acid to be obtained from proteins was leucine. In 1819 it was obtained by a microbiological procedure (1) and in 1820 by acid hydrolysis (2). Almost a hundred years elapsed before the presence was recognized (3) in proteins of an isomer of leucine, isoleucine, which could be separated from leucine only with great difficulty.

With the exception of the protamines, leucine has been found together with isoleucine in all proteins[1] and these amino acids frequently constitute an appreciable part of the molecule. It is only recently that accurate methods have been described for the determination of leucine and isoleucine together[2] and of *l*(+)-leucine.[3, 4] The two methods for the determination of *l*(+)-leucine require much time and analytical skill; the isotope dilution method, moreover, makes use of highly specialized equipment.

This paper describes an accurate, relatively simple microbiological method, based upon the work of Beadle and Tatum on mutants of *Neurospora* (9), for the determination of *l*(+)-leucine. For this purpose, the "*leucineless*" strain of *Neurospora crassa* described by Regnery in the preceding paper (10) was used.

The reliability of the new microbiological procedure was checked by a comparison with the methods dependent on solubility product and the isotope dilution (*cf.* Table V). We are indebted to Dr. Bergmann, Dr. Stein, and Dr. Moore for preparations of gelatin and crystalline egg albumin. With our new method we find 3.6 and 9.6 per cent of leucine respectively in these preparations, while 3.5 and 9.1 per cent were obtained by Moore and Stein by the solubility product method (6). We are likewise indebted to Dr. G. L. Foster for a preparation of crystalline horse hemoglobin with a $l(+)$-leucine content of 15.1 per cent as determined by the isotope dilution method.[5] The leucine content of this preparation determined microbiologically was 15.7 per cent. It, therefore, appears that for proteins of leucine contents ranging from 3 to 16 per cent the microbiological procedure yields values which are consistent with those obtained by two other reliable methods of determination based upon fundamentally different principles.

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* Some of the experiments reported in this paper were presented before the Division of Biological Chemistry at the Pittsburgh meeting of the American Chemical Society, September, 1943.

[1] Ribonuclease does not contain *l*(+)-leucine (E. Brand and F. J. Ryan, unpublished experiments).

[2] Chromatographic adsorption method of Synge and coworkers (4).

[3] Isotope dilution method of Rittenberg and Foster (5) and solubility product method of Bergmann and coworkers (6, 7).

[4] For the designation *cf.* Clarke (8).

A reasonable estimate of the amount of isoleucine in protein hydrolysates may be obtained from the difference between the values for leucine + isoleucine (determined chromatographically (4) or by Dakin's method (11)) and for leucine. For example, the leucine + isoleucine content of gelatin was found to be 6.7 per cent chromatographically (4) and 7.1 per cent by Dakin (11). Subtracting the leucine content of gelatin (3.6 per cent established by two independent methods, *cf.* Table V) we obtain a probable isoleucine content of gelatin of from 3.1 to 3.5 per cent.

The mutant of *Neurospora* which we have used for the determination of leucine needs for growth only this one amino acid, a single vitamin (biotin), sugar, and salts. For bioassays this relative simplicity is a great advantage, particularly when contrasted with the multiple amino acid and vitamin requirements of *Lactobacillus*. This multiplicity and interdependence of growth factors complicates bioassays with various strains of *Lactobacillus*, as is apparent from the current discussion in the literature (12–19). These difficulties are reflected in the discrepancies in the values for the amino acid content of protein hydrolysates determined with the aid of *Lactobacillus*. In a conservative approach we would refrain from considering such microbiological values as significant without other confirmation such as comparison with dependable data in the literature.

In the case of arginine the bioassay with *Lactobacillus casei* gives reliable results since McMahan and Snell's (18) figures for the arginine content of several proteins are in good agreement with the literature. This agreement, however, does not necessarily prove the reliability of this particular procedure (18) when applied to other amino acids; each amino acid constitutes a separate problem.

The interpretation of the microbiological values is particularly difficult for those amino acids for which there are few conclusive data in the literature (*e.g.* the valine content of casein is 6.8 per cent according to McMahan and Snell (18) and 4.9 per cent according to Hegsted (19)).

For the leucine content of proteins reliable data, obtained by the method of solubility product and isotope dilution, are available in the literature only for gelatin, egg albumin, and horse hemoglobin (previous data in the literature refer to the leucine + isoleucine content). Since the method with

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[5] G. L. Foster, personal communication.

Neurospora yields reliable values for the leucine content of the above three proteins (*cf.* Table V), our results can be used for these and other proteins as a basis of comparison. For gelatin (3.6 per cent leucine) and for casein (9.8 per cent leucine) the results of Kuiken *et al.* (17) with *Lactobacillus arabinosus* compare favorably (3.3 and 9.3 per cent leucine respectively). On the other hand, Hegsted (19) finds much less leucine in casein (7.4 per cent) and his value for leucine in edestin (5.5 per cent) is likewise appreciably lower than ours (7.4 per cent).

The fact that Kuiken *et al.* (17) find only 1.7 per cent of isoleucine in gelatin (or one-half of the amount of isoleucine that may, from the above discussion, be assumed to be present) throws doubt upon their value for the isoleucine content of casein (6.5 per cent). From the results for gelatin it could be inferred that their value for casein may be too low, but it seems high if the data in the literature (20) are considered in conjunction with our values for the leucine content of this protein.

The bioassay of amino acids is in its very beginning. Despite the complexities the outlook is favorable, provided that proper criteria are adopted to establish the reliability of the new methods.

EXPERIMENTAL

Organism—The fungus *Neurospora* consists of a white fibrous mat (mycelium) which propagates by the formation of asexual spores (conidia) which appear macroscopically as a fine powder, usually pink, but white in the case of albino strains of *Neurospora*. As the mycelium ages, it becomes transformed almost entirely into conidia which will not germinate; *i.e.*, produce a new mycelium until transferred to fresh medium. The *leucineless* mutant, Strain 33753-4637 (A) (subsequently referred to as the mutant), is an albino, genetically pure and unisexual strain of *Neurospora crassa* and its stock cultures as received are for the most part a conidial mass.

Maintenance of Stock Cultures—Stock cultures are maintained in test-tubes (160 × 16 mm.) on agar slants. These are prepared by dissolving with heat 2 gm. of agar (Bacto) and 0.5 gm. of casein hydrolysate (Casamino acids, Difco) in 100 cc. of basal medium (*cf.* below). About 10 cc. of this solution per test-tube (plugged with non-absorbent cotton) are sterilized by autoclaving for 10 minutes at 15 pounds pressure and allowed to cool in a slanted position. Upon receipt of a stock culture a small clump of conidia is transferred on the tip of a sterile needle to each of several sterile agar slants. Growth is permitted to take place at about 25° for 6 to 8 days until profuse formation of conidia occurs; the test-tubes are then stored at about 10°. Stock cultures prepared and stored in this manner can be used for 1 or 2 months, after which time fresh stock cultures should be prepared by again transferring to fresh medium. In our experience the stock has thus far remained genetically pure and can be maintained if proper sterile technique is used. Contamination of stock cultures has not been observed but if it occurs such cultures should be discarded since purification is rather difficult.

Basal Medium—The basal medium is a modified Fries solution (21) and was selected for reasons discussed in detail by Ryan, Beadle, and Tatum (22); its composition is given in Table I. The basal medium is an almost colorless, *clear* solution with a pH of 5.5. Immediately after the preparation of a batch it is transferred to Pyrex bottles (not larger than 1 liter) which are plugged with non-absorbent cotton and sterilized by steaming in an autoclave first at atmospheric pressure for 15 minutes and then at 15 pounds for 30 minutes, or at 20 pounds for 15 minutes. Repeated autoclaving should be avoided. *Solutions that become opalescent, cloudy, or colored should be discarded.*

TABLE I

*Composition of Basal Medium**

Ammonium tartrate, *gm.*			50
" nitrate, "			10
Potassium phosphate, monobasic, Sörensen's, *gm.*			10
Magnesium sulfate·$7H_2O$, *gm.*			5
Sodium chloride, *gm.*			1
Calcium " anhydrous, *gm.*			1
Sucrose, *gm.*			100
Biotin, S. M. A. Corporation, concentrate No. 200, *cc.*			2 (40 γ)
Boric acid, *mg.*	57	in 1 liter distilled water, *cc.*	10
Ferric chloride·$6H_2O$, *mg.*	96		
Zinc chloride, *mg.*	420		
Manganese chloride·$4H_2O$, *mg.*	144		
Sodium molybdate, *mg.*	42		
Copper sulfate, *mg.*	375		
Distilled water to 10 liters			
Add salts to water			

* All materials are reagent grade.

Assay Technique—The following procedure has been adopted as the result of numerous experiments which will be discussed briefly below but not reported in detail.

1. Up to 5 cc. of a standard solution of *l*(+)-leucine or of an unknown solution such as a protein hydrolysate (*cf.* hydrolysis) are measured into a 125 cc. Pyrex Erlenmeyer flask. If less than 5 cc. of the standard or unknown is used, distilled water is added to make the volume 5 cc. The total amount of leucine in the standard or unknown should be no less than 0.2 and no more than 0.8 mg. (*cf.* "Standardization").

2. 45 cc. of basal medium are added and mixed.

3. The flasks are plugged with non-absorbent cotton and sterilized by autoclaving for 10 minutes at 15 pounds pressure.

4. When cool the flasks are inoculated with conidia from a stock culture. The amount of conidia used is not critical but the smallest quantity macroscopically visible on the tip of a sterile needle should be employed.

5. The flasks are incubated at a constant temperature of 30.0° ± 0.2°.

6. The experimental cultures are permitted to grow for 8½ days, during which time they are shaken by hand twice daily to prevent the mycelium from adhering to the walls of the flask and forming conidia.

7. The contents of the flask are then filtered with suction through a tared 30 cc. glass crucible with a sintered bottom (Jena porosity 4 or Pyrex porosity F, selected for rapid filtration). The tare weight of the crucible is established after it has been cleaned, first with chromate-sulfuric acid solution for 2 hours, rinsed in tap and distilled water, then washed with suction, twice with distilled water, and once with 95 per cent alcohol, and finally dried for 12 hours over $CaCl_2$ in a vacuum desiccator at room temperature. Forceps with rubber tubing on the tips are used to handle the clean crucibles.

8. Suction is continued until the mycelium forms an opaque white mat on the bottom of the crucible; this should not require more than 30 to 60 seconds.

9. The Erlenmeyer flask which contained the mycelium is rinsed twice with 12.5 cc. of distilled water which is used for two washings of the mat in the crucible in such a way that the mat is first stirred up and then reformed as in step (8).

10. The lower part of the crucible is now wiped dry with a clean lint-free cloth.

11. The crucible and contents are dried at room temperature over $CaCl_2$ in a desiccator evacuated with an oil pump (through a drierite chamber) to about 5 mm. pressure. After about 18 hours at this pressure the crucibles have attained constant weight.

12. The crucibles are weighed to within 0.1 mg. and the dry weight of the mycelium determined.

13. The leucine equivalent of the weight of the dry mycelium is read from a standard curve or, preferably, calculated with the aid of a regression coefficient obtained from such a curve.

Although some of the details in the procedure described above may not be critical, any attempted deviation should be carefully considered and an investigation made of the influence of such a change. For instance, the use of a flask different in size and shape from the one recommended in step (1) may influence the results by modifying gas exchange during growth.

Again, the conditions of autoclaving in step (2) are designed so that the sugar concentration is not reduced by caramelization and does not become a limiting factor for growth. No detailed experiments were carried out on the influence of temperature but the 30.0° ± 0.2° recommended in step (5) is not critical. It was chosen because studies on the rate of growth of *Neurospora* in tubes (22) indicate a favorable temperature range between 25–35°. (Unfortunately a temperature of 37° has injurious effects.) At 30°, 43.3 mg. of dry mycelium are produced per mg. of leucine in 8½ days (*cf.* "Standardization").

A number of other points become clear if it is realized that what is measured is approximately maximum growth with leucine as the limiting factor. The amount of mycelium obtained after 7, 8½, and 10 days at 30° was studied and 8½ days was chosen (step (6)) because it lies on the asymptotic part of the growth curve where small differences in time, such as those associated with the filtering procedure, have no detectable effect on mycelial weight. Also, since at 8½ days the mycelium has increased to a good approximation of maximum weight on all concentrations of leucine within the limits of the method (*cf.* "Standardization"), small differences in inoculum size, etc., which affect the *rate* of growth have no influence on final weight. It was therefore not necessary to attempt control of inoculum size by the use of a suspension of conidia.

The determination of dry weight of the mycelium in filter crucibles as described in steps (7) to (12) is a deviation from the technique heretofore employed with *Neurospora* in which the mycelium is pressed between filter paper, rolled into pellets, and dried in an oven.

Standardization—Extensive preliminary experiments were carried out with a commercial preparation of *l*(+)-leucine, the final experiments with a sample of pure *l*(+)-leucine for which we are indebted to Dr. Bergmann, Dr. Stein, and Dr. Moore (*cf.* (6)). In Fig. 1, mg. of mycelial weight are plotted against mg. of leucine present; the individual points represent the average of from two to five determinations in a series of four experiments. It can be seen that a linear relation exists between mycelial weight and leucine, up to about 36 mg. of mycelium. The line in Fig. 1 was calculated by the method of least squares from twenty-four determinations between 11 and 36 mg. of mycelium. The standard deviation of these twenty-four points from the calculated line is 3 per cent and the standard error 0.6 per cent. The relation between mg. of leucine present and mg. of mycelium obtained can therefore be expressed in the form of the following straight line equation,

$$\text{Mg. leucine} = \frac{\text{mg. mycelium}}{42.66} - 0.002 \qquad (1)$$

or approximately

$$\text{Mg. leucine} = 0.02335 \times \text{mg. mycelium} \qquad (2)$$

In assays all experiments yielding mycelial weights of less than 10 mg. are routinely discarded as too small for accurate weighing. Likewise, mycelial weights above 35 mg. are eliminated as too close to the point at which the values begin to deviate from a straight line (*cf.* Table II).

Once a standard curve is established, only occasional checks need be run, since under standard conditions the numerical relation between leucine and growth has proved constant and reproducible over a considerable period of time.

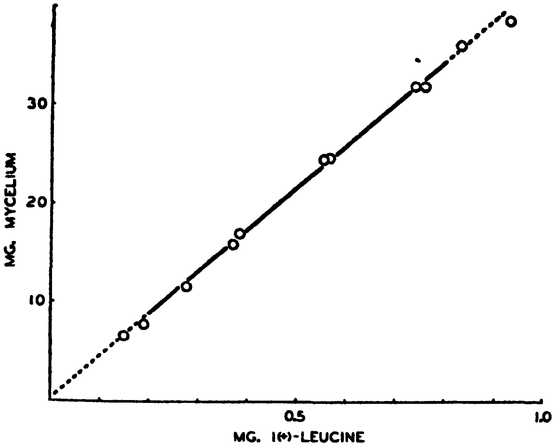

FIG. 1. Relation of leucine to mycelial weight

Adaptations—Occasionally a culture of the mutant grows as if it had no special requirement for $l(+)$-leucine, behaving similarly to the wild type from which it was derived. This phenomenon, as yet not clearly understood, is called "adaptation." Under our standard conditions, the mycelial weight of an "adapted" culture varies from 40 to 100 mg. independently of the amount of leucine present. Such adaptations occur on the average in about 5 per cent of the experiments. They are easily recognized and automatically discarded, since all mycelial weights above 35 mg. are eliminated from consideration (*cf.* "Standardization"). An example is given in Table II, in which the results on insulin are reported. It can be seen that no adaptations occurred in the ten determinations on Hydrolysate 1, while there were three in the ten determinations on Hydrolysate 2.

If an adaptation occurs early in the growth period, it will easily be recognized, but if it occurs near the end, it may result in only a slight increase in

TABLE II

*Determination of l(+)-Leucine in Crystalline Insulin**

Hydrolysate No. (1)	Hydrolysate analyzed: Volume (2)	Hydrolysate analyzed: Protein (3)	Weight of dry mycelium after 8½ days (4)	Leucine equivalent of dry mycelium calculated from standard leucine curve (5)	Leucine content of protein (6) = $\frac{(5)}{(3)} \times 100$
	cc.	mg.	mg.	mg.	per cent
1†	1.0	0.91	4.8‡		
	1.0	0.91	4.7‡		
	1.5	1.37	6.5‡		
	1.5	1.37	6.5‡		
	2.0	1.83	10.2	0.237	12.9
	2.0	1.83	9.9‡		
	3.0	2.74	15.3	0.356	13.0
	3.0	2.74	16.0	0.373	13.6
	4.0	3.64	21.8	0.509	14.0
	4.0	3.64	21.7	0.507	13.9
Mean ± standard error					13.5 ± 0.3
Standard deviation					0.5
2§	1.5	2.15	12.1	0.283	13.1
	1.5	2.15	11.3	0.264	12.3
	1.5	2.15	11.6	0.272	12.6
	2.0	2.89	16.7	0.390	13.5
	2.0	2.89	17.3	0.404	14.0
	2.0	2.89	43.6‡		
	2.0	2.89	45.2‡		
	3.0	4.31	25.5	0.596	13.8
	3.0	4.31	24.6	0.574	13.3
	3.0	4.31	51.3‡		
Mean ± standard error					13.2 ± 0.3
Standard deviation					0.6
1 + 2	Combined mean ± standard error (12 determinations)				13.3 ± 0.2
	Standard deviation				0.6

Leucine content of crystalline insulin (corrected for ash) = 13.4 %, 101.4 moles per gm. × 10^6, 47 residues per mole of 46,000 (ultracentrifuge, *cf.* (23)), 45 residues per mole of 44,600 (from unpublished analytical data of E. Brand and L. J. Saidel) mycelial weight. Such partial adaptations are probably not much more frequent than complete adaptations (*e.g.*, there were none in the twenty determinations on insulin hydrolysates in Table II). The partial adaptations cannot be distinguished from high values obtained for other reasons and therefore must be eliminated by a statistical analysis of the data (*cf.* Table III).

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* We are indebted to Dr. V. du Vigneaud for the sample of crystalline insulin (ash content 0.8 per cent).

† 36.55 mg. of dry protein were hydrolyzed for 13 hours with 3 cc. of 6 N HCl in an oil bath at 130–140°. The hydrolysate was adjusted with 2.5 N NaOH to pH 5.2 (glass electrode), brom-cresol green being used as an internal indicator, and made up to 100 cc.

‡ Only mycelial weights between 10 and 35 mg. are considered (*cf.* "Standardization").

§ 35.94 mg. of dry protein were hydrolyzed for 15½ hours in the same way as Hydrolysate 1, but made up to 25 cc. (pH 4.30).

Statistical Analysis—The mean and standard deviation of the per cent leucine are calculated for the individual hydrolysates (Table III, Column 6). All values which deviate from the mean by more than twice the standard deviation are eliminated. In view of the occurrence of partial adaptations it is statistically justifiable to eliminate such values. This is essentially the same as accepting as significant a probability of 0.05, a procedure widely used by statisticians (*cf.* (24)). The accepted values from two or more hydrolysates (with similar means) are then combined, the mean and standard deviation established, and all values eliminated which differ by more than twice the standard deviation. For instance, in the experiments with horse hemoglobin, reported in Table III, three out of eight values are eliminated from those for Hydrolysate 1, while for Hydrolysate 2 only two out of eight differ from the mean by more than twice the standard deviation. In establishing the combined mean for Hydrolysates 1 and 2, another value from Hydrolysate 1 is eliminated. The final value for horse hemoglobin, based on ten determinations, is 15.7 ± 0.1 per cent with a standard deviation of 0.2 per cent, in close agreement with the leucine content obtained by the isotope dilution method[5] (15.1 per cent, *cf.* Table V). It should be noted that in the case of hemoglobin all values eliminated by statistical analysis are higher than the mean finally established. This is in agreement with our general experience in which values lower than the mean by more than twice the standard deviation are rarely observed. This, in turn, confirms our suspicion that high values are frequently the result of a partial adaptation. In order to obtain significant results, it seems necessary to carry out a considerable number of determinations (eight to twelve) per hydrolysate and eliminate statistically any partial adaptations.

The values in Tables II to VI are presented as means ± their standard errors.

Hydrogen Ion Concentration—The dependence of the growth of the mutant on pH has not been studied in detail under our conditions (*cf.* (10)). The growth of the mutant begins on a medium (basal + leucine standard) with a pH of 5.5; when growth is completed, after 8½ days, the pH is about 4.5. If the acidity of the 5 cc. of unknown used in the assay (*cf.* "Assay technique," step (1)) is between pH 1.5 and 2.2, somewhat high values of mycelial weight are obtained. It has been our impression that higher

TABLE III

*Determination of l(+)-Leucine in Horse Hemoglobin**

Hydrolysate No.	Hydrolysate analyzed		Weight of dry mycelium after 8½ days	Leucine equivalent of dry mycelium calculated from standard leucine curve	Leucine content of protein $(6) = \frac{(5)}{(3)} \times 100$
	Volume	Protein			
(1)	(2)	(3)	(4)	(5)	
	cc	*mg.*	*mg.*	*mg.*	*per cent*
1†	2	1.81	12.1	0.282	15.6
	2	1.81	11.9	0.277	15.3
	3	2.73	22.7‡	0.530‡	(19.4)‡
	3	2.73	19.5‡	0.455‡	(16.7)‡
	3	2.73	18.3	0.427	15.6
	4	3.65	24.6	0.575	15.8
	4	3.65	25.7	0.600	16.4§
	4	3.65	26.0‡	0.608‡	(16.7)‡
Mean ± standard error					15.7 ± 0.2
Standard deviation					0.4
2‖	2	1.87	12.9	0.300	16.0
	2	1.87	13.5‡	0.314‡	(16.8)‡
	3	2.80	19.3	0.451	16.1
	3	2.80	18.7	0.436	15.6
	3	2.80	18.9	0.441	15.8
	4	3.76	25.4	0.593	15.8
	4	3.76	25.1	0.586	15.6
	4	3.76	26.5‡	0.619‡	(16.5)‡
Mean ± standard error					15.8 ± 0.1
Standard deviation					0.2
1 + 2	Combined mean ± standard error (10 determinations)				15.7 ± 0.1
	Standard deviation				0.2

Leucine content of horse hemoglobin = 15.7 %, 119.7 moles per gm. × 10^6, 80 residues per mole (66,700)

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[*] We are indebted to Dr. G. L. Foster for the sample of recrystallized horse hemoglobin.

[†] 90.6 mg. of dry protein were hydrolyzed for 14 hours with 7 cc. of 6 N HCl in an oil bath at 130-140°. Most of the HCl was removed by repeated (four times) evaporation *in vacuo;* the hydrolysate was then made up to a volume of 100 cc. and filtered.

[‡] These values are excluded since they deviate from the mean by more than twice the standard deviation. They are possibly the result of a partial adaptation of the *leucineless* mutant to the wild type condition.

[§] This value is excluded since it deviates from the combined mean by more than twice the standard deviation.

[‖] 93.7 mg. of dry protein were hydrolyzed for 18 hours and treated essentially as Hydrolysate 2. The pH of the final solution was 3.4 (glass electrode).

acidities may be associated with a somewhat higher incidence of complete and partial adaptations. Although this point has by no means been established, it is desirable to adjust the pH of the unknown to between 3.5 and 5.0. Since brom-cresol green has no influence on the final weight of the mutant, it can be used as an internal indicator to adjust the pH of the hydrolysate to about 4.5.

Preparation of Hydrolysates—The hydrolysis is carried out in flasks with a capacity of 40 to 75 cc., connected to reflux condensers by a standard glass joint (19/38). Several small boiling stones are added; capryl alcohol should be avoided. From 25 to 250 mg. of protein are hydrolyzed with 3 to 10 cc. of 6 N HCl for 13 to 17 hours in a bath kept at 130–140°. About 90 per cent of the HCl is neutralized with the calculated amount of 2.5 N NaOH with cooling; then 5 to 15 drops of 0.02 per cent solution of brom-cresol green are added and the hydrolysate is adjusted to approximately pH 4.5 by addition of dilute NaOH. For strongly colored hydrolysates pH indicator paper is also used. After neutralization the hydrolysate is made up to 25 to 100 cc. and filtered. Aliquots are set up immediately for leucine determinations as described in step (1) of the assay procedure and the rest of the hydrolysate used for checking the pH.

TABLE IV

Effect of Time of Hydrolysis on Leucine Values of Casein and Egg Albumin

Protein	Per cent of leucine				
	Time of hydrolysis				
	3 hrs.	8 hrs.	14–16 hrs.	26 hrs.	30–34 hrs.
Casein, vitamin-free, Labco.....	9.2 ± 0.1	9.6 ± 0.2	9.8 ± 0.1	8.5 ± 0.1	8.2 ± 0.1
No. of hydrolysates .	1	1	5	1	1
Egg albumin (Bg)*. ..	8.8 ± 0.1	9.7 ± 0.1	9.6 ± 0.1		8.5 ± 0.2
No. of hydrolysates...	1	1	2		2
Egg albumin (C), crystalline, denatured. .			9.9 ± 0.1		
No. of hydrolysates...			2		

* Obtained from Dr. Bergmann, Dr. Moore, and Dr. Stein (*cf.* (6)).

In our earlier experiments HCl was removed by repeated evaporation *in vacuo* (*cf.* Table III), but the neutralization procedure above is simpler and usually allows a better adjustment of the final pH.

A preliminary hydrolysis is carried out and an approximate estimate of the leucine content obtained by setting up a series of determinations in which the amount of protein is widely varied (*cf.* Table II). For subsequent assays the amount of protein to be hydrolyzed and the final volume of the neutralized hydrolysate are chosen so that there are present between

0.1 and 0.2 mg. of leucine per cc. of diluted hydrolysate. About eight to twelve individual determinations at two to four levels of leucine (preferably between 0.25 and 0.75 mg.) are run with between 1 and 5 cc. of hydrolysate (*cf.* Tables II and III).

Time of Hydrolysis—In Table IV are presented some experiments with casein and egg albumin in which the time of hydrolysis was varied from 3 to 34 hours. It can be seen that after 8 and 14 to 16 hours of hydrolysis the values for the leucine content did not differ statistically. However, hydrolysis for 3 hours and for 26 to 34 hours gives significantly lower results. Hydrolysis for about 13 to 16 hours was therefore adopted as a routine procedure.

Results

In seven experiments in which known amounts of *l*(+)-leucine were added to casein hydrolysates an average recovery of 101.5 per cent was obtained.

Similar experiments in which *d*(−)-leucine[6] (0.24, 0.48, and 0.96 mg.) was added showed a uniform utilization of 17 per cent of this sample at all three levels. Our observations on the utilization by *Neurospora* of the unnatural isomer of leucine in the presence of *l*(+)-leucine agree with those of Regnery (10). The inefficiency of such utilization when *l*(+)-leucine is not present has been described by Regnery in the preceding paper and the possible rôle of *d*-amino acid oxidase discussed (10). These findings are of interest in connection with observations on the rat. Ratner *et al.* (25) found that under normal conditions the unnatural isomer was freely utilized, whereas Rose (26) found that the utilization of *d*(−)-leucine was insufficient for the growth of young rats when *l*(+)-leucine was rigidly excluded from the diet.

Experiments with *dl*-isoleucine showed no utilization of this compound by the mutant in the presence of protein hydrolysates.

The leucine content of gelatin, egg albumin, and horse hemoglobin is reported in Table V. It can be seen that the results obtained by the three methods, *viz.* the solubility product method, the isotope dilution method, and the *Neurospora* microbiological method, are in substantial agreement. Our value for egg albumin is slightly higher than that obtained by Moore and Stein (6). However, we hydrolyzed only for about 15 hours with HCl, while the results with the solubility product method are based on hydrolysis for 30 hours with HCl in the presence of $SnCl_2$ (*cf.* Table IV).

In Table VI, some preliminary data on the leucine content of skim milk powder, dried yeast, and wheat flour are presented. For comparison, we

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[6] We are indebted for the *d*(−)-leucine to S. Ratner and D. Rittenberg; for the optical rotation *cf.* (25). This preparation may have contained small amounts of *l*(+)-leucine (*cf.* (25)).

TABLE V

Comparison of Leucine Content Obtained by Different Methods

Protein	Hydrolysis	Method	Leucine
			per cent
Gelatin (Bg)[*]	14–16 hrs., HCl	*Neurospora*	3.6
" (O)[†]	14–16 " "	"	3.6
" (Bg)[*]	30 hrs., HCl-$SnCl_2$	Solubility product	3.5
Egg albumin (Bg)[*]	14–16 hrs., HCl	*Neurospora*	9.6
" " (C)	14–16 " "	"	9.9
" " (Bg)[*]	30 hrs., HCl-$SnCl_2$	Solubility product	9.1
Horse hemoglobin[‡]	14–16 hrs., HCl	*Neurospora*	15.7
" "	18 hrs., HCl	Isotope dilution[§]	15.1

[*] We are indebted for these preparations to Dr. Bergmann, Dr. Stein, and Dr. Moore (6); for the values obtained by the solubility product method *cf.* (6).

[†] We are indebted for this gelatin, prepared by the late Dr. T. B. Osborne, to Dr. H. B. Vickery.

[‡] We are indebted to Dr. G. L. Foster for the preparation of horse hemoglobin.

[§] This result was obtained by G. L. Foster (private communication) by the isotope dilution method (5).

TABLE VI

Analysis of Foodstuffs and Related Proteins

Material	Leucine	Tryptophane[*]	Tyrosine[*]
	per cent	*per cent*	*per cent*
Skim milk powder[†]	3.5	0.47	2.06
Casein	9.8	1.20	6.10
β-Lactoglobulin[‡]	15.4	1.94	3.72
Dried yeast[§]	2.9	0.54	1.80
Wheat flour[‖]	0.8	0.07	0.37
Gliadin[¶]	6.5	0.66	3.14

[*] The values for tryptophane (ultraviolet absorption of tryptophane mercurial) and tyrosine were obtained by E. Brand and L. J. Saidel (unpublished experiments) (*cf.* (27)).

[†] The values are not corrected for moisture and ash. 251 mg. were hydrolyzed with 12 cc. of 6 N HCl for 15 hours at 130–140°. The hydrolysate was adjusted to pH 4.3 with 18 N and with dilute NaOH, made up to 50 cc., and filtered.

[‡] The β-lactoglobulin was obtained from Dr. R. K. Cannan.

[§] The values are not corrected for moisture and ash. 251 mg. were hydrolyzed essentially the same way as the skim milk (final volume 50 cc., pH 3.7).

[‖] The values are not corrected for moisture and ash. 1.22 gm. were hydrolyzed with 10 cc. of 6 N HCl + 1 cc. of concentrated HCl for 23 hours at 130–140°. The hydrolysate was adjusted to pH 4.5 with 18 N and with dilute NaOH, made up to 50 cc., and filtered.

[¶] Gliadin was obtained from Dr. H. B. Vickery.

have included data on the tyrosine and tryptophane content of these materials and also the values on proteins present in these materials. The tryptophane determinations were carried out with a new method, based upon the absorption in the ultraviolet (at 254 mμ) of the tryptophane mercurial (27).

The question of the possible loss of leucine in the humin remains to be studied. However, Kuiken *et al.* (17) found little loss of leucine in sulfuric acid hydrolysates of casein to which large amounts of carbohydrate had been added.

The work reported in this paper was made possible through the kindness of Dr. George W. Beadle, who put the "*leucineless*" strain of *Neurospora* at our disposal.

Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

SUMMARY

A microbiological method for the determination of leucine in protein hydrolysates and foodstuffs is described.

The *leucineless* strain of *Neurospora crassa* reported by Regnery in the preceding paper was used.

The reliability of this method has been established by a comparison with the methods involving solubility product and isotope dilution. For gelatin and egg albumin we find microbiologically a leucine content of 3.6 and 9.6 per cent respectively, while the results on the identical preparations by the solubility product method were 3.5 and 9.1 per cent. For horse hemoglobin we find 15.7 per cent of leucine, while 15.1 per cent was obtained on the identical preparation by the isotope dilution method.

The influence of the time of hydrolysis was studied. Maximum results are obtained after about 15 hours of hydrolysis.

The leucine content of crystalline insulin and edestin, the leucine, tryptophane, and tyrosine contents of casein, β-lactoglobulin, skim milk powder, dried yeast, gliadin, and wheat flour are reported.

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A MICROMETHOD FOR THE DETERMINATION OF ACETONE AND KETONE BODIES

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(Received for publication, March 24, 1944)

The method for the determination of acetone in small amounts of biological fluids and air presented here was developed primarily for a study of absorption and elimination of acetone under conditions comparable to industrial exposure to acetone vapors (1). The method, however, has useful clinical application, since it can be adapted for the rapid estimation of ketone bodies as well as acetone in small amounts of blood and urine.

The concentrations of acetone in the air which have been proposed as safe for industrial exposure of 8 hours range from 0.5 to 5.0 mg. per liter (2–4); the maximum concentrations of acetone developed in the blood are of the order of 1.5 to 30.0 mg. per cent. With the method of analysis described in the literature it is impossible, at these levels, to follow with accuracy from small samples of blood the rise and fall of the concentration in the blood during and after exposure. The repeated withdrawal of blood, especially in experiments in which small animals are employed, precludes the use of amounts larger than can be obtained from skin puncture.

The most sensitive methods reported in the literature for the determination of acetone and ketone bodies, with 0.2 cc. of blood, are the nephelometric method of Shipley and Long (5) and the iodometric method of Weichselbaum and Somogyi (6). The first of these methods is, according to the authors, sensitive to 1.0 to 1.5 mg. per cent of ketone bodies with a possible error of 15 per cent. Weichselbaum and Somogyi, however, have pointed out that at low concentrations it may yield results which are several hundred per cent too high and that it is difficult to obtain consistent results. The second method is accurate but is sensitive to only 10 mg. per cent of ketone bodies and requires 3 to 4 hours for a single determination. Both of these methods necessitate distillation of the acetone with possibility of some loss.

The method reported here is rapid, requires no distillation, and is 10 times more sensitive than previous methods. With 0.2 cc. of blood in the determination of acetone, the method responds to 0.1 mg. per cent with an error of ±0.05 mg. per cent; and in the determination of total ketone bodies, to 0.2 to 0.3 mg. per cent expressed as β-hydroxybutyric acid with an error of ±0.12 mg. per cent. In the determination of acetone in air the absolute sensitivity is 0.1 to 0.2 γ with an error of ±0.06 γ; thus if a sample of 1 liter is used, as in the analysis of room air containing acetone, the sensitivity is 0.04 to 0.08 part per million.

The method reported here is based upon the reaction of acetone with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone, the separation of the hydrazone by extraction with carbon tetrachloride, and its colorimetric determination in the carbon tetrachloride. Dakin and Dudley (7) first noted that certain hydrazones, owing to their differential solubilities, could be fractionated with alcohol and that the hydrazones gave intense colors in NaOH solution. These observations have since been applied by a number of investigators (8–15) to the determination of substances containing a true carbonyl group, particularly pyruvic acid. The application to keto acids, in general, and a study of the differential solubilities of their hydrazones are given by Friedemann and Haugen (16).

In the procedures previously described for keto acids the hydrazone is extracted from the solvent and its color measured in alkaline solution. The solvents were selected on the basis of the ease with which they extracted the hydrazone and released it to the alkaline solution. Friedemann and Haugen found that carbon tetrachloride was unsuited for the extraction of pyruvic acid hydrazone. It has been found here, however, that carbon tetrachloride readily extracts acetone hydrazone from acid solution and gives up little on reextraction with alkali. Because of these solubility characteristics it is possible to eliminate interference from keto acids and estimate the yellow acetone hydrazone directly in the carbon tetrachloride. This feature is especially advantageous since the color complex formed by the acetone hydrazone with alkali is unstable.

Acetaldehyde 2,4-dinitrophenylhydrazone is largely extracted from the carbon tetrachloride by alkali and the concentration of acetaldehyde occurring in the blood causes no interference. In the determination of acetone in air, as in industrial plants, large amounts of aldehyde or other ketones may cause interference. Formaldehyde, however, causes no interference because its hydrazone is completely extracted by the alkali.

In the determination of acetone in air, blood, or urine, and ketone bodies in blood or urine, various initial steps are carried out as described in subsequent sections; the final common step is the reaction of acetone with a constant volume of 2,4-dinitrophenylhydrazine solution. The hydrazone formed is then extracted with a constant volume of carbon tetrachloride which is then extracted with alkali. These extractions and the colorimetric determination of the acetone hydrazone are described and discussed here as the common feature of all the procedures to be subsequently described.

Reagents—

1. Acid 2,4-dinitrophenylhydrazine solution; 0.1 per cent 2,4-dinitrophenylhydrazine in 2.0 N HCl.

2. Carbon tetrachloride, reagent.
3. Sodium hydroxide solution, 0.5 N.

2 cc. of acid 2,4-dinitrophenylhydrazine solution are placed in a glass-stoppered graduate of 10 cc. capacity; the solution containing the acetone to be analyzed is added to give a total volume of 5 cc. Exactly 2 cc. of carbon tetrachloride are added. The graduate is stoppered and placed in a mechanical shaker for 10 minutes. The supernatant acid solution is then drawn off, the graduate is filled twice with distilled water which is also drawn off each time, and 3 cc. of 0.5 N NaOH solution are added. It is again shaken for 3 minutes. The contents of the graduate are then poured into a Klett micro colorimeter tube and read against a similar tube containing carbon tetrachloride, a Klett Filter 42 which transmits light of wave-length 420 mμ being used. From this reading is subtracted that of a blank determination in which distilled water is used instead of the solution for analysis; this blank results from impurities in the reagents and the retention of a small amount of hydrazine in the carbon tetrachloride. It remains constant for any one preparation of the reagents.

The amount of acetone hydrazone recovered in the carbon tetrachloride is less than the total amount formed from the reaction of acetone with the hydrazine. This recovery is influenced by two features: (1) the volumes of the fluids involved in the extractions; (2) the strength of the acid and alkali solutions used. Both of these features were investigated here in so far as they affected the analytical procedures. Purified acetone 2,4-dinitrophenylhydrazone was used. 2 cc. of a solution of this hydrazone in carbon tetrachloride which gave a reading of 111 divisions on the Klett colorimeter were first extracted with 3 cc. of 0.5 N NaOH. The color density of the carbon tetrachloride did not change; a second and third extraction with 3 cc. of 0.5 N NaOH caused each time a 5 per cent decrease in the reading. The explanation offered here for lack of change in the color density after the first extraction is that the color of the hydrazone is enhanced by the presence of alkali and that this enhancement compensates for the small amount of hydrazone extracted by the alkali. A second 2 cc. portion of the hydrazone in carbon tetrachloride solution was extracted with 5 cc. of 1.3 N HCl solution. A reading of 96 divisions was obtained, indicating a loss of 13.5 per cent of the hydrazone owing to its solubility in the acid solution. Finally, a third 2 cc. portion of hydrazone in carbon tetrachloride solution was extracted with 5 cc. of 0.04 per cent 2,4-dinitrophenylhydrazine in 1.3 N HCl, washed with distilled water, and extracted with 3 cc. of 0.5 N NaOH. After the blank obtained by similar extraction of carbon tetrachloride alone was subtracted, a reading of 123 divisions was obtained. Thus in spite of the loss of hydrazone due to acid and alkaline extraction and over and above the enhancement of color due to the presence of alkali there was a further enhancement of the color of the hydrazone due to the presence of hydrazine. Thus, when the extractions involved in the analytical procedures described in the present investigation are carried out, there is a loss of 13.5 per cent of the hydrazone in the extraction with 0.04 per cent hydrazine in 1.3 N acid, a loss of 5 per cent in the extraction with 0.5 N alkali, a 30 per cent enhancement of the color of hydrazone in carbon tetrachloride due to the hydrazine, and, finally, an additional 5 per cent enhancement of color due to the presence of alkali. Because of the complicated interplay of losses of hydrazone due to extraction and enhancement of color in each extraction, it was impractical to standardize the analytical method by measuring only the photometric density of solutions of known amounts of acetone hydrazone in carbon tetrachloride. Repeated determinations showed that, by using constant volumes of fluids in the extractions and maintaining constant normalities of the acid and alkali solutions, constant and reproducible readings were obtained from known amounts of acetone. Furthermore, various amounts of the hydrazone gave readings which followed Beer's law.

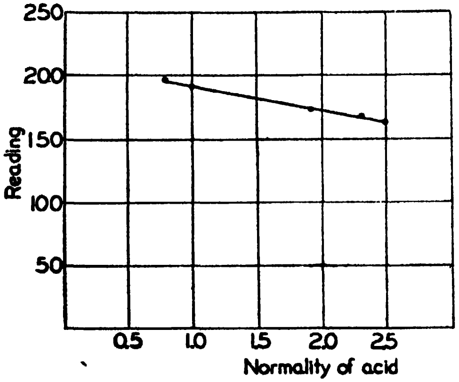

FIG. 1. Relation of acidity to the recovery of acetone hydrazone

The strength of the alkaline solution used in extracting the carbon tetrachloride in the analytical procedures described here was uniformly 0.5 N; losses at other strengths were not determined quantitatively; it was noted, however, that alkali of greater strength extracted more hydrazone. In determinations made in air, blood, and urine the strength of acid used in the preliminary steps in preparing the hydrazone varied; the influence of these variations on recovery was therefore studied. 2 cc. portions of a solution of acetone hydrazone in carbon tetrachloride were extracted first with acid hydrazine and then with alkali; the acidity of the acid hydrazine solution was varied from 0.8 N to 2.5 N. The recoveries obtained are shown in Fig. 1 in which the photometric density of the hydrazone expressed as divisions read on the colorimeter is plotted against the normality of the acid. The points obtained fall on a straight line of which the slope is expressed by the equation, $R = 210 - 21N$, in which R is the photometric density and N is the normality of the acid. Thus with each $1N$ increase of the acid there is a 10 per cent increase of the hydrazone extracted from the carbon tetrachloride and the photometric densities at various normalities of acid have the following relationship to each other.

$$\frac{R}{1 - 0.1N} = \frac{R_1}{1 - 0.1N_1} = \frac{R_2}{1 - 0.1N_2}$$

This relationship holds for all concentrations of hydrazone within the range of normalities studied.

Determination of Acetone in Air

3 cc. of the 2,4-dinitrophenylhydrazine solution are added to a tube or flask in which the air sample is to be collected. The tube is evacuated with a water pump and the negative pressure read from a mercury manometer. The volume of the sample to be drawn is calculated as V in the equation

$$V = \frac{(V_1 - S)(P_1 - P_2)273}{760(273 + t)}$$

in which V_1 is the total volume of the air-sampling tube, S the volume of the hydrazine solution, P_1 and P_2 the prevailing barometric pressure and the residual pressure in the tube after evacuation in mm. of mercury. The tube is opened in the air of which the concentration of acetone is to be determined; after being closed, it is shaken for 1 or 2 minutes. The acetone admitted to the tube is rapidly taken up by the hydrazine solution and converted to the hydrazone. 1 or 2 cc. of the solution are transferred to a 10 cc. glass-stoppered graduate; if only 1 cc. is used, an additional 1 cc. of the acid hydrazine is added to maintain the desired volume. 3 cc. of water are added to bring it to a volume of 5 cc. The solution in the graduate is then treated as described under the general procedure and the color density read from the colorimeter. From this value the blank is subtracted and from the remainder the acetone is calculated.

For this calculation it is necessary to standardize the colorimeter for known amounts of acetone; this standardization serves for all subsequent similar determinations. 3 cc. of an accurately prepared acetone solution containing 2 to 4 mg. per liter of distilled water are added to 2 cc. of the acid hydrazine solution in a 10 cc. glass-stoppered graduate and subjected to the general procedure described. The reading of the colorimeter after the blank is subtracted, is divided into the weight, in mg. of acetone in the sample used; the result is taken as factor F, which is mg. of acetone per division as read from the colorimeter. This factor, once determined, remains constant for the colorimeter.

To permit accurate reading of the Klett colorimeter, the largest amount of hydrazone in the carbon tetrachloride should correspond to no more than 0.02 mg. of acetone; *i.e.*, a reading of approximately 300 on the colorimeter. Thus for air containing from 0.1 to 0.5 mg. per liter, the sampling tube should have a capacity, exclusive of the 3 cc. of hydrazine solution added, of not more than 120 cc. if 1 cc. of this solution is to be used for analysis and 60 cc. if 2 cc. are to be used; for higher or lower concentrations, the volumes should be proportionately smaller or larger.

TABLE I

Recovery of Acetone from Air Containing Known Amounts

Acetone added	Acetone found	Error	
		Average	Extremes
mg. per 1000 cc.	*mg. per 1000 cc.*	*per cent*	*per cent*
3.02	3.03		
	3.01		
	2.99		
	3.05	0	+1.0 to −1.0
1.57	1.56		
	1.59		
	1.55		
	1.55	−0.5	+1.3 " −1.3
0.68	0.68		
	0.69		
	0.68		
	0.66	−0.4	+1.5 " −2.9
0.27	0.26		
	0.25		
	0.27		
	0.27	−2.6	+0 " −7.4

The concentration of acetone as mg. per liter in the air is calculated from the equation

$$\text{Mg. acetone per liter air} = \frac{1000(R - B)FD}{V}$$

in which R is the reading, B the blank, F the standardization factor, D the volume of hydrazine solution put in the sampling tube divided by the volume taken out for analysis, and V the volume of the air sample. Values obtained from the analyses of air containing known amounts of acetone are shown in Table I.

Determination of Acetone in Blood and Urine

0.2 cc. of blood or urine is delivered into 1 cc. of water in a 10 cc. test-tube and 3 cc. of 5 per cent trichloroacetic acid are added. The mixture is filtered or centrifuged in a stoppered tube and 3 cc. of clear fluid are put in a 10 cc. glass-stoppered graduate. 2 cc. of the acid hydrazine solution are added and the solution treated as described under the general procedure. If froth develops during shaking, it can be removed before reading by a few minutes of centrifuging in the colorimeter tube. The blank is determined by using 0.2 cc. of distilled water instead of blood or urine. The standardization for factor F is made by adding 1 cc. of an accurately prepared acetone solution containing 8 to 16 mg. per liter to 3 cc. of 5 per cent trichloroacetic acid, putting 3 cc. of this mixture, containing the known amount of acetone, in a 10 cc. glass-stoppered graduate, and proceeding as above. The concentration of acetone as mg. per cent in the blood or urine is calculated from the equation

$$\text{Mg. acetone per 100 cc.} = \frac{420F(R - B)}{0.6}$$

Values obtained from the analyses of water, blood, and urine containing known amounts of acetone are shown in Table II. The normal blood and urine used to make these solutions showed no acetone on preliminary analysis.

Determination of Total Ketone Bodies in Blood and Urine

All current methods for the determination of ketone bodies in blood and urine are based upon the conversion of acetoacetic acid to acetone by acid hydrolysis and the oxidation of β-hydroxybutyric acid to acetone with acid dichromate. The acetone is then separated either by distillation or by precipitation as a complex mercury salt. In the distillation of small amounts of acetone, Shipley and Long (5) were unable to avoid losses. These were attributed by them to an unexplained disappearance of acetone when boiled in solution in a sealed glass vessel. Although Weichselbaum and Somogyi (6) lost no acetone in distillation, they obtained a loss of acetone in the precipitation with Denigès' reagent owing to some solubility of this precipitate. A careful study was carried out by these authors as to the optimum concentrations of sulfuric acid and dichromate for the oxidation of β-hydroxybutyric acid to acetone. In their procedure for the determination of ketone bodies, oxidation of the β-hydroxybutyric acid and distillation are carried out simultaneously; they point out, however, that the ideal conditions would be those under which the concentrations of acid and dichromate did not change.

Since it is not necessary to separate acetone in preparation for its reaction with 2,4-dinitrophenylhydrazine, it was possible in the method reported here to use a micro refluxing tube in the conversion of the ketone bodies to acetone and to avoid distillation. Thus there was no change in the concentration of acid and dichromate. This apparatus, shown in Fig. 2, consists of a Pyrex glass tube 20 cm. long and 2 cm. in diameter, terminating in a standard taper female glass grinding. Around the outside of this tube, from approximately 3 cm. above the bottom to just below the glass grinding, is a water jacket. Inside the tube is a cold finger 1.5 cm. in diameter which extends to 3 cm. above the bottom and whose upper end is fused into a tapered male glass grinding so that it fits tightly into the opening of the tube. The effluent arm of the outside water jacket is connected by a rubber tube to the inlet arm of the cold finger so that during operation cold water flows through both the jacket and the inner tube. This rubber tube is disconnected for removal of the cold finger. 3 cc. of fluid placed in this refluxing apparatus with a few glass beads or an ebullition tube can be brought quickly to active boiling, which can then be main-

TABLE II

Recovery of Acetone from Water, Blood, and Urine Containing Known Amounts

Water				Blood				Urine			
Acetone added	Acetone re-covered	Error		Acetone added	Acetone re-covered	Error		Acetone added	Acetone re-covered	Error	
		Average	Extremes			Average	Extremes			Average	Extreme
mg. per 100 cc.	*mg per 100 cc.*	*per cent*	*per cent*	*mg per 100 cc.*	*mg. per 100 cc.*	*per cent*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*per cent*
9.90	9.83			9.92	9.88			9.01	9.07		
	9.92				9.97				8.99		
	9.87		+0.7		9.88				8.99		+0.7
	9.97	0.0	−0.7		9.97		+0.5		8.94	−0.1	−0.8
4.95	5.03				9.88	0.0	−0.4	5.03	4.98		
	4.89			5.01	5.04				5.02		
	4.98		+1.6		4.95				4.93		+0.0
	4.94	+0.2	−1.2		4.98		+0.6		4.98	−1.0	−2.0
1.98	1.98				4.95	−0.6	−1.2	1.17	1.15		
	2.07			1.03	1.06				1.19		
	1.98		+4.5		1.01				1.19		+1.7
	2.07	+2.3	−0.0		1.01				1.15	0.0	−1.7
					1.01						
					1.06		+2.9				
					1.06	+0.5	−1.9				
				0.48	0.44						
					0.48						
					0.48		+10.4				
					0.53	+0.5	−8.3				

tained for any length of time in a completely closed system. Solutions containing even very low concentrations of acetone boiled in the apparatus for 30 minutes showed no loss.

The studies of Shaffer and Marriott (17), Van Slyke (18), and Weichselbaum and Somogyi (6) emphasize the effect upon the products of the oxidation of β-hydroxybutyric acid caused by change in the concentration of dichromate and sulfuric acid. In the development of the present technique, the yield of acetone from the oxidation of β-hydroxybutyric acid was studied with various concentrations of sulfuric acid and dichromate. 3 cc. portions of solutions containing known amounts of the Ca-Zn salt of β-hydroxybutyric acid[1] were refluxed with various concentrations of acid and dichromate in the apparatus described above. The acetone content of the mixture was then determined from reaction with 2,4-dinitrophenylhydrazine as described, but with one additional step necessitated because of the presence of dichromate. Dichromate destroys both hydrazone and

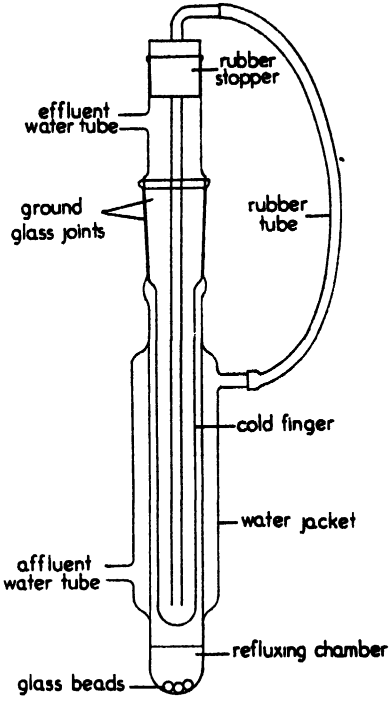

FIG. 2. Micro refluxing apparatus

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[1] Supplied through the courtesy of the Scripps Metabolic Institute, La Jolla, California.

hydrazine; therefore a few drops of a solution of sodium sulfite are added to the mixture to be analyzed before the addition of the hydrazine solution. The resulting chromium sulfate does not interfere with the subsequent analysis. Consistent maximum yields of 86 per cent were obtained with an acid concentration of 2.6 N in the reaction mixture and a dichromate concentration of 0.077 per cent. Both the concentration of acid and dichromate and the yield are in agreement with those of Weichselbaum and Somogyi. Known amounts of ethyl acetoacetate were similarly refluxed with the same concentrations of sulfuric acid and dichromate. The yield of acetone was virtually 100 per cent. Both the oxidation of β-hydroxybutyric acid and the hydrolysis of ethyl acetoacetate were found to be complete in 10 minutes.

For the preparation of a deproteinized blood filtrate, zinc sulfate and barium hydroxide were used as described by Weichselbaum and Somogyi (6). These precipitants do not interfere with the reaction of acetone and 2,4-dinitrophenylhydrazine as does tungstic acid. Trichloroacetic acid cannot be used, since it is easily oxidized and utilizes an appreciable amount of the dichromate, thus affecting the dichromate concentration.

Weichselbaum and Somogyi pointed out that sugars and lactic acid, on treatment with sulfuric acid and dichromate, yield materials which form precipitates with Denigès' reagent. Neither of these was found to yield substances reacting with 2,4-dinitrophenylhydrazine. In a group of normal human beings, Weichselbaum and Somogyi found total ketone bodies in the blood, expressed as β-hydroxybutyric acid, ranging from 0.25 to 0.94 mg. per cent. In the present investigation, refluxing of normal blood filtrates with the concentrations of acid and dichromate described above gave total ketone values 3 to 5 times as high as these. It was therefore concluded that interfering substances were produced by hydrolysis or partial oxidation from materials other than sugar and lactic acid in normal blood filtrate. Further investigation showed, however, that these products could be more completely oxidized and removed as interfering substances after the oxidation of β-hydroxybutyric acid was completed by an additional short period of refluxing with stronger dichromate. This did not destroy any of the acetone already formed. Thus the blood filtrate was refluxed for 10 minutes with acid and weak dichromate, more dichromate added, and the mixture refluxed for an additional 10 minutes. Complete analyses with this procedure for the conversion of ketone bodies to acetone gave, in normal bloods, total ketone bodies, expressed as β-hydroxybutyric acid, ranging from 0.36 to 1.20 mg. per cent in seventeen subjects. These values are of the same order of magnitude as those found by Weichselbaum and Somogyi. Urine may be treated as blood for the determination of ketone bodies.

The reagents required in addition to those for the general procedure are (1) zinc sulfate solution, approximately 2.5 per cent zinc sulfate ($ZnSO_4 \cdot 7H_2O$); (2) barium hydroxide solution, approximately 0.15 N. This solution must be so adjusted that when 5 cc. of the zinc sulfate solution are titrated with it (phenolphthalein as indicator) 5 cc. are required to produce a permanent pink color; (3) acid dichromate solution, 0.46 per cent potassium dichromate in 15.6 N H_2SO_4; (4) 10 per cent potassium dichromate solution; (5) 15 per cent anhydrous sodium sulfite solution.

0.2 cc. of blood or urine is added to 2.3 cc. of distilled water in a small centrifuge tube. The blood is allowed to lake and 1 cc. of barium hydroxide solution is added. The contents of the tube are mixed and 1 cc. of zinc sulfate solution added. The contents of the tube are again mixed and the tube stoppered and centrifuged for 2 to 3 minutes. 3 cc. of the supernatant fluid are delivered to the bottom of the clean, dry refluxing tube (Fig. 1) containing several glass beads or a small ebullition tube; 0.6 cc. of acid dichromate solution is added, and the tube tightly stoppered with the cold finger. The rubber tube from the water jacket to the cold finger is connected and the flow of cold water through the water jacket and cold finger started. The contents of the reflux tube are heated by means of a micro gas burner and gentle boiling is continued for 10 minutes. At the end of this time the burner is removed and when boiling has ceased the cold finger is lifted just enough to allow the introduction of 0.5 cc. of 10 per cent dichromate solution from a 1 cc. hypodermic syringe. This solution is allowed to run down the cold finger. The refluxing tube is then closed tightly and boiling continued for an additional 10 minutes. At the end of this time the flow of water through the apparatus is discontinued and the entire tube is cooled under a water tap. The tube is inverted several times so that any material that might have condensed on the upper walls of the tube will be washed down. The cold finger is removed and the contents of the reflux tube are ready for determination of acetone.

3 cc. of the fluid in the reflux tube are delivered into a 10 cc. glass-stoppered graduate. 0.4 cc. of sodium sulfite solution is added from a burette to destroy the dichromate. From this point the procedure described under the general section is followed.

The blank, *B*, which must be subtracted from the reading obtained, is determined as follows: To 3 cc. of distilled water are added 0.6 cc. of the acid dichromate solution and 0.5 cc. of the 10 per cent dichromate solution. 3 cc. of this mixture are then carried through the same procedures as the 3 cc. portion of filtrate after it has been refluxed.

In standardization of the readings of the colorimeter for factor *F*, 0.6 cc. of the acid dichromate solution and 0.5 cc. of the 10 per cent dichromate solution are added to 3 cc. of a carefully prepared acetone solution containing 3 to 5 mg. of acetone per liter. 3 cc. of this mixture, containing a known amount of acetone, are then delivered into a 10 cc. graduate and treated by the procedure as described above. The concentration of acetone derived from the ketone bodies in the blood or urine is obtained from the equation

$$\text{Mg. acetone per 100 cc. blood or urine} = \frac{1845F(R - B)}{1.8}$$

This concentration of acetone does not represent the true concentration of ketone bodies, since only 86 per cent of the hydroxybutyric acid is oxidized to acetone; an exact estimation can be made only if the proportion of β-hydroxybutyric acid present is known. It has been shown by Weichselbaum and Somogyi (6) that normally the ketone bodies, β-hydroxybutyric acid and acetoacetic acid plus acetone, are present in the equivalent parts of approximately 2:1. On the basis of this distribution a recovery of 91 per cent can be used for calculation and the value obtained in the last equation is divided by 0.91. The results may be expressed as equivalent amounts of acetoacetic acid or β-hydroxybutyric acid. Recoveries of known amounts of β-hydroxybutyric acid added to water, blood, and urine are shown in Table III.

TABLE III

Recovery of β-Hydroxybutyric Acid from Water, Blood, and Urine Added in Known Amounts

Water				Blood				Urine			
Acid added	Acid found	Error		Acid added	Acid found	Error		Acid added	Acid found	Error	
		Average	Extremes			Average	Extremes			Average	Extremes
mg per 100 cc	*mg. per 100 cc*	*per cent*	*per cent*	*mg. per 100 cc.*	*mg per 100 cc.*	*per cent*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*	*per cent*
2 79	2.84			0	2.12			0	2.96		
	2.60		+1.8	2.45	4.48			2.45	5.32		
	2.72	−2 5	−6.8		4.48		+6.1		5.44		+6.1
8.66	8.63				4.72	−0.4	−3.7		5.56	+1.2	−3.7
	8.51		+1.0	9.80	11.82			9.80	12.76		
	8.75	−0.3	−1 7		12.05		+1.3		12.66		0.0
					11.94	+0.2	−1.0		12.66	−0.7	−1.0

The determination of β-hydroxybutyric acid alone may be made by eliminating acetone and acetoacetic acid from the blood or urine filtrate preliminary to the oxidation of the β-hydroxybutyric acid. 0.2 cc. of blood or urine is deproteinized as described above for the determination of total ketones; the filtrate is poured into a small Pyrex test-tube with a few glass beads or an ebullition tube and 1 drop of concentrated sulfuric acid is added. The tube and contents are weighed and are then boiled for 5 minutes. In this length of time the acetoacetic acid is completely hydrolyzed and all of the acetone is boiled off. The tube is then cooled and its original weight restored by the careful addition of distilled water. 3 cc. of this fluid are then treated in the same way as 3 cc. of the original filtrate for the determination of total ketone bodies. The same blank and factor, *F*, are used as in the determination of total ketone bodies. The concentration of β-hydroxybutyric acid, expressed as acetone, is obtained from the equation given above, by dividing by 0.86 instead of 0.91. To express the result as β-hydroxybutyric acid the value obtained is multiplied by 1.79. The value of 0.86 is used instead of 0.91 in the above equation because the only ketone body present in this analysis is β-hydroxybutyric acid which yields only 86 per cent acetone in oxidation. From separate determinations of total ketone bodies and β-hydroxybutyric acid the acetone plus acetoacetic acid may be calculated by difference. From a third determination of the acetone alone a value for each of the three components of the ketone bodies may be obtained.

SUMMARY

A rapid, sensitive and accurate method for determining acetone in air and biological fluids is described. The method is based upon the reaction of acetone with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone, the separation of the hydrazone by extraction with carbon tetrachloride, and its colorimetric determination in this fluid. The method, with modifications described, affords rapid and accurate determination of the total and of individual ketone bodies.

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AROMATIC SULFONIC ACIDS AS REAGENTS FOR PEPTIDES. PARTIAL HYDROLYSIS OF SILK FIBROIN

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(Received for publication, April 4, 1944)

The isolation of pure peptides from the products of the partial hydrolysis of a protein involves the fractionation of relatively complex mixtures of peptides and amino acids. The salts of peptides with aromatic sulfonic acids crystallize well, are frequently less soluble than are the corresponding free peptides, and exhibit, in some cases, wide differences in solubility. The use of arylsulfonic acids for the isolation and determination of peptides has, therefore, been suggested (1). The present communication concerns the application of arylsulfonic acids as reagents for the isolation of dipeptides from a hydrolysate of silk fibroin.

Before such an isolation was attempted, the approximate solubilities of the arylsulfonates of several peptides were determined. The results are presented in Table I. The peptides selected include some of those which may be expected to result from the partial hydrolysis of silk fibroin. In addition four peptides containing leucine or phenylalanine have been included because the solubility of their arylsulfonates may become of interest in future studies of proteins other than fibroin. Glycine, alanine, tyrosine, and serine are appended since these four amino acids may be formed during the partial hydrolysis of fibroin.

It is of interest that glycyl-*l*-leucine and glycyl-*l*-phenylalanine resemble leucine and phenylalanine in that they both form sparingly soluble salts with all the sulfonic acids tested. The salts of *l*-leucylglycine and *l*-leucylglycylglycine, on the other hand, are much more soluble.

Before the information given in Table I was used for the isolation of dipeptides from a fibroin hydrolysate, the course of the hydrolysis of fibroin by acid was investigated in order to establish conditions under which maximal quantities of dipeptides are formed. For this purpose the hydrolysis of fibroin was followed by both the nitrous acid and ninhydrin methods of Van Slyke. This experimental approach has been illustrated by Van Slyke, Dillon, MacFadyen, and Hamilton for the differentiation of peptides and free amino acids in tryptic digests of casein (3). In the special case of the acid hydrolysis of silk fibroin, it is possible to calculate not only the quantity of peptides present at any given time, but also the average peptide chain length. It is essential to this calculation that the amounts of NH_2-N and COOH-N obtained after complete hydrolysis of fibroin are each equivalent to about 96 per cent of the total N of the protein. This result is consistent with the fact that proline, hydroxyproline, lysine, and aspartic acid either do not occur in silk fibroin, or are present only in very small amounts. The 4 per cent of non-NH_2-N in the hydrolysate is accounted for by arginine and ammonia.

Data on the course of the hydrolysis of fibroin by concentrated hydrochloric acid at 40° are given in Table II. The NH_2-N figure (Column 3) provides a measure of the free amino groups present in peptides and amino acids. The amount of COOH-N (Column 4) is a measure of the amino acids present, since peptides do not evolve CO_2 with ninhydrin. Subtraction of the amino acid value (Column 4) from the total NH_2-N gives the amount of peptide NH_2-N (Column 5). The nitrogen present in peptides (Column 6) is equal to 96 per cent of the total N of the hydrolysate (Column 2) minus the COOH-N (Column 4). Thus at a given time during the hy-

TABLE I

Approximate Solubility Products of Arylsulfonates of Several Peptides and Amino Acids

The solubilities were determined at 0° in the manner already described (2). Where no value is given, the solubility product of the salt in question was found to be greater than about 4 × 10^{-2}. The values listed are × 10^{-4}.

Peptides and amino acids	Sulfonic acid										
	Naphthalene-β-	2-Bromotoluene-5-	5-Nitronaphthalene-1-	Flavianic	O-(2,4-Dinitrophenyl)-*p*-phenol-	*p*-Bromobenzene-	2,5-Dibromobenzene-	2,6-Diiodophenol-4-	2-Nitro-4-iodophenol-	Azobenzene-*p*-	*p*-Hydroxyazobenzene-*p*'-
Glycyl-*l*-leucine	20	20	20	1	6	200	100	200	6	0.3	0.4
l-Leucylglycine											
l-Leucylglycylglycine											
Glycyl-*l*-phenylalanine	4	4	4	0.1	4	20	4	50	4	0.1	0.4
Glycyl-*l*-tyrosine	20			0.7						0.5	0.5
l-Tyrosylglycine					20						0.2
Glycylglycine	50	50	20	20	50		20	50		2	
Glycyl-*l*-alanine			20	50	20		3	6	20	3	3
l-Alanylglycine			4		20			20			
l-Alanyl-*l*-alanine					20						
Glycine			5	25						4	10
l-Alanine										2	20
l-Tyrosine				0.03	2	200	20	8	2		1
l-Serine											2

drolysis the average peptide chain length (Column 7), in terms of amino acid residues, is equal to the nitrogen present in peptides (Column 6) divided by the NH_2-N (Column 5) of the peptides.

It will be noted that in the course of the hydrolysis the longer peptide chains are rapidly degraded during the first 3 hours to an average length of about 6 amino acid residues. Between 40 and 48 hours the chain length levels off at the dipeptide stage. Even at 96 hours the value in Column 7 has not decreased below 2. This fact supports the validity of the determinations employed in the calculation of the values in Column 7. In this connection it may be pointed out again that the values in Column 7 represent the average chain length of the peptides only, and not the average chain length of the sum of the peptides and amino acids.

TABLE II

Hydrolysis of Silk Fibroin by Concentrated HCl at 40°, Measured by Nitrous Acid and Ninhydrin Methods

Time of hydrolysis (1)	Total N of hydrolysate (2)	NH_2-N (3)	COOH-N (4)	NH_2-N in peptides (5)	Nitrogen in peptides (6)	Average peptide length (7)
hrs	*mM*	*mM*	*mM*	*mM*	*mM*	
0.5	2.17	0.07	0.004	0.07	2.08	29.7
1.5	2.42	0.26	0.020	0.24	2.30	9.6
3.0	2.48	0.47	0.063	0.41	2.32	5.7
6.0	2.52	0.69	0.109	0.58	2.31	4.0
10.0	2.47	0.92	0.187	0.73	2.18	3.0
17.2	2.47	1.17	0.278	0.89	2.09	2.3
24.0	2.47	1.26	0.408	0.85	1.96	2.3
33.0	2.47	1.37	0.507	0.86	1.86	2.2
42.7	2.47	1.50	0.666	0.83	1.70	2.05
48.0	2.47	1.48	0.641	0.84	1.73	2.06
96.0	2.47	1.71	1.005	0.70	1.36	1.95

The percentage of free amino acid molecules present in the hydrolysate at a given time is illustrated graphically in Fig. 1. This percentage is calculated according to the expression (mM CO_2/(0.96 × mM total N)) × 100.

The amino nitrogen values have been calculated as per cent of complete hydrolysis. As will be noted from Fig. 1, the rate of amino nitrogen production is rapid during the initial hours and tapers off as the peptide chain length approaches 2. The production of free amino acids, however, continues at a slow, steady rate.

After 40 hours of hydrolysis the fibroin hydrolysate contains approximately 25 per cent free amino acids and 75 per cent peptides. As will be seen from Table II, these peptides are almost exclusively dipeptides. The hydrolysate obtained after 40 hours was, therefore, regarded as optimally suited to the isolation of dipeptides, and was used for this purpose in the experiments to be described later.

The validity of the foregoing calculations depends upon the applicability of both the nitrous acid and ninhydrin methods to this problem. There is no evidence to indicate that the ninhydrin method yields erroneous results when applied to fibroin hydrolysates. It is known, however, that glycyl dipeptides give high amino nitrogen values even in the presence of potassium iodide. Thus, Kendrick and Hanke (4) obtained 120 per cent of theory for glycylglycine (in the presence of KI) and we find that glycylalanine gives 108 per cent under the same conditions. Alanylglycine, on the other hand, reacts normally. Calculation of the maximum amount of glycyl dipeptides which could be expected in the 40 hour hydrolysate indicates that a possible error of 2 to 4 per cent in the value for the amino nitrogen may be involved. This would correspond to an error of 0.2 to 0.3 in the average peptide length, which for the present investigation would not significantly alter the conclusions.

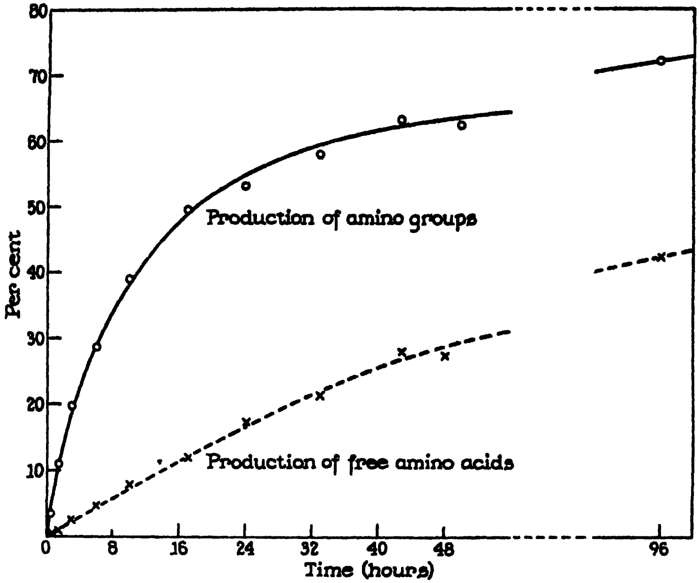

FIG. 1. Rate of hydrolysis of silk fibroin at 40° (2 cc. of concentrated HCl per gm. of fibroin).

The fact should be mentioned here that several workers (for references see (5)) have reported the isolation of diketopiperazines after hydrolysis of fibroin under conditions practically identical with those used in this investigation. However, the isolation procedures employed by these workers were such that diketopiperazines might have been formed as artifacts. The data in Table II and Fig. 1 clearly indicate that after silk fibroin has been hydrolyzed under the conditions used in these studies, for 30 hours or longer, the hydrolysate cannot contain appreciable amounts of diketopiperazines. If present, they would increase the non-NH_2-N to such an extent that the average peptide chain length could not approach the value of 2 as closely as it does.

The isolation of dipeptides from partial hydrolysates of fibroin is facilitated by the fact that glycine and alanine residues comprise the major part of the protein. Thus, dipeptides containing these two amino acids should predominate in the partial hydrolysate. In fact, earlier investigators have already isolated *l*-alanylglycine (6) and β-naphthalenesulfonylglycyl-*l*-alanine (7). The isolation of the free dipeptide, glycyl-*l*-alanine, has not been previously reported. Abderhalden has also obtained glycyltyrosine (8), and claimed the isolation of diketopiperazines containing serine (9).

The results of the preliminary investigations described in the foregoing paragraphs permitted the formulation of a simple working procedure for the selective precipitation of sulfonates of the two isomeric dipeptides containing glycine and *l*-alanine. Glycyl-*l*-alanine was first precipitated as the salt of 2,5-dibromobenzenesulfonic acid, and subsequently *l*-alanylglycine was precipitated as the salt of 2,6-diiodophenol-4-sulfonic acid. The yield of each of these dipeptides was 6 gm. per 100 gm. of fibroin.

Amino Acid Composition of Fibroin—In an earlier publication from this laboratory (10) it was stated that glycine was one-half, alanine one-fourth, and tyrosine one-sixteenth of all the amino acids in silk fibroin. The average residue weight of all the amino acid residues in silk fibroin was assumed to be 84. Synge (5) and Chibnall (11) have pointed out that the average residue weight of 84 is far too high and, consequently, that the frequencies given by Bergmann and Niemann for glycine, alanine, and tyrosine must be in error. There can be little doubt that these criticisms of Synge and of Chibnall are valid, and that the average residue weight of the amino acid residues in silk fibroin is about 75 to 76. We had quite independently come to the same conclusions as the above mentioned investigators, and accordingly had instituted a reexamination of the amino acid content of silk fibroin. Since there seems little likelihood at present of concluding these investigations, it appears worth while to place on record our preliminary results in order to correct some of the earlier data of Bergmann and Niemann.

It seems probable that the value given by Bergmann and Niemann for the alanine content of fibroin is far too low, and the tyrosine value is appreciably too high.

Several lines of evidence point to the probability that silk fibroin contains between 30 and 35 per cent alanine. In the first place, preliminary analyses by the solubility product method indicated an alanine content in this range. These analyses were performed at a time when the solubility method had not reached its present stage of development (12), and with a reagent (sodium dioxpyridate) (13) not well suited to this purpose. Nevertheless, the results, though only approximate, were entirely incompatible with the value of 26 per cent given by Bergmann and Niemann. In the second place, pure *l*-alanine has been isolated from hydrolysates of silk fibroin in a yield of 24 per cent (2). If the value of Bergmann and Niemann is correct, this would represent a recovery of over 92 per cent of all the alanine in the hydrolysate. In view of the nature of the isolation procedure, so high a yield seems virtually impossible. It is very probable, therefore, that there is appreciably more than 26 per cent alanine in fibroin.

One further consideration also points to the presence of more than 26 per cent alanine in fibroin. If the value for alanine was 26 per cent, then about 85 per cent of the nitrogen of the protein could be accounted for at present, leaving about 15 per cent of the nitrogen unaccounted for.[1] The very low average residue weight of the amino acids in silk, coupled with the substantial tyrosine content (about 12 per cent), requires that part, at least, of this unknown nitrogen be contributed by amino acids of low molecular weight (glycine, alanine, or serine). Glycine cannot comprise any of this unknown fraction, since the glycine content of 44 per cent is well established (12). Serine also can be ruled out as a major constituent of this unknown fraction, since the present serine value, though perhaps not definitely settled, cannot be far in error. By process of elimination, therefore, it appears probable that a substantial part of the nitrogen of silk fibroin as yet unaccounted for will prove to be alanine nitrogen.

Each of the three independent lines of evidence discussed above, no one of which is decisive of itself, points to the same conclusion; namely, that there probably is considerably more than 26 per cent of alanine in silk fibroin.

Preliminary analyses for *l*-tyrosine in which the original procedure of the solubility method (15) was employed gave a value of 11.9 per cent.[2] Flavianic acid was employed as reagent. Although this value has not been checked sufficiently to certify its accuracy, we feel that it is more accurate than the value of 13.3 per cent reported by Bergmann and Niemann. The figure 11.9 per cent is also in agreement with the value 12.1 per cent reported by Rutherford, Patterson, and Harris (16), although it is higher than the value of 10.8 per cent reported by Meyer *et al.* (17).

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[1] Synge (5) states that 90 per cent of the nitrogen of silk is accounted for. In making this calculation, Synge assumes that fibroin contains 18.5 per cent nitrogen, and employs the value obtained by him, 17.4 per cent, for serine. The fibroin analyzed by us, however, contained 19.0 per cent nitrogen. Moreover, Nicolet and Shinn (14) reported several values for the serine content of fibroin, all of which were lower than the one reported by Synge.

[2] In the determination of *l*-tyrosine by the solubility method, *d*-tyrosine, or *dl*-tyrosine, if present, would not be determined.

EXPERIMENTAL

Partial Hydrolysis of Silk Fibroin—Japanese white silk, degummed in this laboratory with papain-H_2S, was employed in this investigation. The silk fibroin contained 6.2 per cent water (determined by drying over P_2O_5 at 100° *in vacuo* for 24 hours), 0.08 per cent ash as sulfate, and 19.0 per cent nitrogen (ash- and moisture-free). To each of a series of 0.200 gm. samples of this material in 10 cc. volumetric flasks, 0.40 cc. of concentrated HCl was added, and the flasks were maintained at 40°. After varying time intervals, the hydrolysis was stopped by the addition of 5 cc. of cold water and sufficient 4 N NaOH to render the medium alkaline to Congo red and acid to litmus. Each sample was diluted to 10 cc., the slight precipitate which formed was centrifuged off, and the clear solution stored at 0° with thymol. Aliquots were removed for determination of total N, NH_2-N, and COOH-N. The results are summarized in Table II. The NH_2-N determinations were performed with the addition of 2 per cent KI to the acetic acid (4) in all cases. The COOH-N determinations were performed on 2 cc. aliquots of the hydrolysate to which 100 mg. of citrate buffer and 150 mg. of ninhydrin were added, according to the procedure of Van Slyke, Dillon, MacFadyen, and Hamilton (3).

Solubilities of Peptide Salts of Aromatic Sulfonic Acids—The approximate solubility products of the peptide salts listed in Table I were obtained in the same manner as were the approximate solubility products of the amino acid arylsulfonates (2). The peptides used in this investigation were all prepared according to directions to be found in the literature,[3] with the exception of *l*-alanyl-*l*-alanine. The preparation of this compound, by the carbobenzoxy method, is described below.

Synthesis of l-Alanyl-l-alanine Carbobenzoxy-l-alanyl-l-alanine Ethyl Ester—Carbobenzoxy-*l*-alanine, prepared as described by Bergmann and Zervas (19), was converted to the acyl chloride according to the procedure outlined by Hunt and du Vigneaud (20). A dry chloroform solution of carbobenzoxy-*l*-alanyl chloride prepared from 3.3 gm. of carbobenzoxy-*l*-alanine was mixed at about −50° with one-half of a chloroform solution of *l*-alanine ethyl ester prepared from 1.5 gm. of *l*-alanine. An aqueous solution of 1.48 gm. of $KHCO_3$ was added with shaking, followed by the second half of the ester solution. The mixture was kept at −10° for 10 minutes and at room temperature for 1 hour. The chloroform solution was extracted with dilute HCl, bicarbonate, and water, dried over Na_2SO_4, and concentrated under reduced pressure. For crystallization the residue was dissolved in ethyl acetate and petroleum ether was added. Yield, 2.9 gm.; m.p., 114–115°. For analysis the product was recrystallized from ethyl acetate and petroleum ether; m.p., 116°.

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[3] In repeating the synthesis of *l*-alanylglycine by the carbobenzoxy procedure, it was found that carbobenzoxy-*l*-alanylglycine, when recrystallized from either water (preferable) or ethyl acetate, had a melting point of 132–133° instead of 103–104° as reported previously (18).

$C_{16}H_{22}O_5N_2$.	Calculated.	C 59.6, H 6.8, N 8.7
322	Found.	" 59.4, " 6.7, " 8.7

Carbobenzoxy-l-alanyl-l-alanine—A solution of 2.0 gm. of the ethyl ester in 10 cc. of acetone and 7.0 cc. of N NaOH was allowed to stand at room temperature for 40 minutes. After the addition of 7.6 cc. of N HCl the product crystallized readily during the removal of the acetone under reduced pressure. Yield, 1.7 gm.; m.p., 152–153°. For analysis the substance was recrystallized from water.

$C_{14}H_{18}O_5N_2$.	Calculated.	C 57.1, H 6.1, N 9.5
294	Found.	" 57.0, " 6.2, " 9.6

l-Alanyl-l-alanine—The carbobenzoxy compound was dissolved in methanol, hydrogenated with palladium as the catalyst, and the free dipeptide isolated in the usual manner. After one recrystallization from water and alcohol the compound had the rotation of $[\alpha]_D^{24} = -21.7°$. Fischer (21) reported $[\alpha]_D^{20} = -21.6°$ for *l*-alanyl-*l*-alanine.

Isolation of Glycyl-l-alanine and l-Alanylglycine—Technically degummed Japanese white silk (533 gm.) was incubated at 40° for 44 hours with 1060 cc. of concentrated HCl. The bulk of the HCl was removed by adding a solution of 2.2 kilos of lead acetate trihydrate (analytical reagent) and the volume of the filtrate and washings was recorded. The lead remaining in the solution was precipitated as sulfide. The approximate amount of HCl remaining in the solution was calculated from the solubility of lead chloride in the volume of solution used, and enough concentrated HCl was added so that the solution contained about equimolar amounts of NH_2-N and HCl. The hydrolysate was concentrated to about 2 liters and stored at 0°. The solution contained 44.5 mg. of nitrogen per cc., corresponding to 234 mg. of protein per cc.

To 250 cc. of this hydrolysate 80 gm. of 2,5-dibromobenzenesulfonic acid were added and the solution was stored at 0° for 4 days. The precipitate which formed was filtered off, washed with a little water, and dried. Yield, 22 gm. The filtrate, Solution A, was saved for the isolation of *l*-alanylglycine. This glycyl-*l*-alanine dibromobenzenesulfonate was contaminated with one or more impurities which were difficult to remove.

One or two recrystallizations of the salt did not accomplish sufficient purification, for the dipeptide obtained by decomposition of the recrystallized salt exhibited a low rotation of $[\alpha]_D^{27} = -42.6°$ (10 per cent in water). Nor could the impurity be removed by recrystallization of the free dipeptide. Pure glycyl-*l*-alanine was obtained, however, by purification through the use of a second sulfonic acid, which gives a sparingly soluble salt with the peptide. For this purpose, 2,6-diiodophenol-4-sulfonic acid may be used, since separation from alanylglycine has already been accomplished. To 22 gm. of the impure dibromobenzenesulfonate dissolved in 75 cc. of hot water, a hot solution of 13.8 gm. of barium acetate monohydrate in 25 cc. of water was added. The mixture was cooled to 0°, and the barium dibromobenzenesulfonate was filtered off and washed with water. To the filtrate and washings, 4.5 cc. of concentrated H_2SO_4 were added, the $BaSO_4$ removed by filtration, and 25 gm. of 2,6-diiodophenol-4-sulfonic acid were added. The solution was stored at 0° overnight, whereupon glycyl-*l*-alanine 2,6-diiodophenol-4-sulfonate crystallized in large prisms. The salt was filtered off, washed with water, and dried. Yield, 13 gm. An additional 4 gm. were obtained by concentrating the mother liquor to 40 cc. The salt thus obtained (17 gm.) was recrystallized twice from water. The yield was 12.6 gm., corresponding to 3.2 gm. of glycyl-*l*-alanine or 5.5 per cent of the silk employed.

$C_6H_4O_4SI_2 \cdot C_5H_{10}O_3N_2$.	Calculated.	C 23.1, H 2.5, N 4.9
572	Found.	" 23.0, " 2.8, " 4.8

Free glycyl-*l*-alanine was obtained from the salt in the following manner. To 11.4 gm. of salt dissolved in hot water, a hot solution of 5.5 gm. of barium acetate monohydrate was added. The mixture was cooled, the barium 2,6-diiodophenol-4-sulfonate filtered off and washed with water, and the barium removed from the filtrate and washings with exactly the required quantity of sulfuric acid. The solution was concentrated to dryness under reduced pressure, the residue was taken up in water, and alcohol added to induce crystallization. A yield of 2.4 gm. of glycyl-*l*-alanine was obtained, corresponding to 83 per cent of the theory, calculated on the basis of the salt employed. The material was recrystallized once from water-alcohol for analysis.

$C_5H_{10}O_3N_2$.	Calculated.	C 41.1, H 6.85, N 19.2
146	Found.	" 41.25, " 6.8, " 19.1

$[\alpha]_D^{27} = -48.6°$ (10% in water)

Fischer and Schulze (22) reported $[\alpha]_D^{20} = -50.0°$ for synthetic glycyl-*l*-alanine.

To the filtrate, Solution A, from which glycyl-*l*-alanine dibromobenzenesulfonate had been removed, 100 gm. of 2,6-diiodophenol-4-sulfonic acid were added. After standing several days at 0°, the precipitated salt was filtered off and recrystallized once from 35 cc. of water. The yield of *l*-alanylglycine 2,6-diiodophenolsulfonate was 14.6 gm., corresponding to 3.5 gm. of *l*-alanylglycine or 6 per cent of the silk employed.

$C_6H_4O_4SI_2 \cdot C_5H_{10}O_3N_2 \cdot 2H_2O$. Calculated. C 21.7, H 3.0, N 4.6
608 Found. " 21.7, " 3.1, " 4.6

The salt was decomposed with barium acetate and the free dipeptide obtained in the manner already described for the preparation of glycyl-*l*-alanine. The yield of pure dipeptide was 92 per cent of the theory calculated on the basis of the salt employed.

$C_5H_{10}O_3N_2$. Calculated. C 41.1, H 6.85, N 19.2
146 Found. " 41.1, " 6.9, " 19.0
$[\alpha]_D^{27} = +50.0°$ (4% in water)

Fischer (23) reported $[\alpha]_D^{18} = +50.3°$ for *l*-alanylglycine.

SUMMARY

1. The solubility of a series of peptide salts of aromatic sulfonic acids has been investigated as an illustration of the applicability of these reagents to the isolation of peptides.

2. The course of hydrolysis of silk fibroin by concentrated HCl at 40° has been followed by both the Van Slyke nitrous acid and ninhydrin methods, permitting calculation of the average peptide chain length at any given time.

3. From a partial hydrolysate containing dipeptides, glycyl-*l*-alanine and *l*-alanylglycine have been isolated by the successive use of 2,5-dibromobenzenesulfonic acid and 2,6-diiodophenol-4-sulfonic acid.

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THE EFFECT OF EXCESSIVE NICOTINAMIDE FEEDING ON RABBITS AND GUINEA PIGS

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(Received for publication, March 24, 1944)

Slightly depressed growth and fatty livers were observed in animals fed moderate amounts of nicotinamide, while larger quantities completely inhibited growth (1, 2). The fatty livers were found to be susceptible to the action of choline, while only methionine was found to alleviate the growth inhibition. These phenomena were ascribed to the forced synthesis of trigonelline from nicotinamide with consequent diminution of the "labile methyl" supply, or, more specifically, the supply of methionine. It has since been demonstrated (3) that the methylated excretory product resulting from nicotinamide feeding is not trigonelline but N^1-methylnicotinamide (F_2). However, it was not determined whether the effects of nicotinamide feeding were due solely to the diminution in the "labile methyl" supply or to toxicity of the excess, unmethylated nicotinamide.

In the present work it has been found that the ingestion of large quantities of nicotinamide by young rabbits and guinea pigs does not impair their growth. Since neither of these species excreted extra N^1-methylnicotinamide after nicotinamide feeding, it is concluded that the toxic effects of nicotinamide in the rat are due entirely to the synthesis of N^1-methylnicotinamide and consequent depletion of available methionine.

EXPERIMENTAL

Rabbits—Two diets were employed in this series. Diet A was a commercial rabbit chow in pellet form[1] used as a stock feed for our colony. Nicotinamide was incorporated therein by spraying a shallow layer of pellets with the proper volume of a 10 per cent solution and then drying in a current of warm air. Diet B was a mixture of oats, rye, alfalfa, grass, and other dry vegetable matter and nicotinamide was added to this ration in the same manner. All animals were housed in individual cages and were offered hay *ad libitum* every 5th day. Weanling rabbits, average weight 1250 gm., were used and after 20 days the animals were sacrificed by decapitation and samples of liver taken for fat analysis. Urinary N^1-methylnicotinamide excretion was determined by the method of Huff and Perlzweig (4) on the pooled urines of three rabbits collected for 48 hours on the 15th and 16th days of the experiments. These measurements were very kindly performed by Mr. Jesse W. Huff of the Biochemistry Department. The results are summarized in Table I. It will be seen that the presence of nicotinamide in either diet had no significant effect on the animals' growth, liver fat content, or urinary N^1-methylnicotinamide excretion.

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[1] Purina chow, Ralston Purina Company, St. Louis, Missouri.

As controls, young rats were also fed Diet A. These results are also presented in Table I. They agree completely with the observations previously obtained with synthetic diets (1). No data are presented for the N^1-methylnicotinamide excretion of rats, as this has already been done in detail (1). For comparison with the results obtained herein with rabbits and guinea pigs it may be stated that under comparable circumstances the presence of 2 per cent nicotinamide in rat diets produced an 80-fold increase in N^1-methylnicotinamide (thought to be trigonelline at the time) excretion.

TABLE I

Effect of Nicotinamide Feeding on Rabbits

Basal diet	Test animal	Supplement to basal diet	Weight change	Liver fatty acids	F$_2$ excretion
			gm. per day	*per cent*	*γ per day*
A	Rabbits	None	25.3	4.5	740
"	"	1% nicotinamide	22.1	4.3	790
"	"	2% "	20.9	3.9	600
"	Rats	None	2.9	5.2	*
"	"	1% nicotinamide	1.1	11.4	*
"	"	2% "	−0.6	3.1	*
B	Rabbits	None	21.0	3.5	1030
"	"	1% nicotinamide	17.3	3.9	970
"	"	2% "	22.5	3.6	1120

* Complete data have already been published (1).

Guinea Pigs—The basal diet used in this series was patterned after that of Woolley (5). The composition of Basal Diet 1 was as follows: casein 20, sucrose 45, cod liver oil 5, cottonseed oil 10, salt mixture (6) 5, and linseed meal 15. The linseed meal was extracted three times at room temperature with a 75:25 mixture of ether and alcohol to remove some of the choline, since seed meals have been reported to be a rich source of this substance (7). Each kilo of diet also contained thiamine 3 mg., riboflavin 5 mg., pyridoxine 3 mg., nicotinic acid 10 mg., pantothenic acid 25 mg., and ascorbic acid 100 mg. Basal Diet 2 was similar to this but contained in addition, 0.15 per cent of choline and of inositol. The animals were offered the experimental diets *ad libitum* 7 days after birth. Their average weight at the start of the experiment was 124 gm. N^1-Methylnicotinamide values were calculated from determinations performed on the pooled urine of three guinea pigs collected for 48 hours on the 16th and 17th days of the experiment. The results are summarized in Table II. All the animals ate sparingly for the first 5 days on the experimental diets. Accordingly, the figures presented in Table II were determined for a period of 3 weeks following the 5 days of relative anorexia.

Again the presence of nicotinamide appeared to exert no toxic effect. No significant disparities in growth, liver fat content, or N^1-methylnicotinamide excretion were noted.

TABLE II

Effects of Nicotinamide Feeding on Guinea Pigs

Diet	Weight change	Liver fatty acids	F_2 excretion
	gm. per day	*per cent*	*γ per day*
Basal Diet 1	1.2	4.3	110
" " 1 + 1% nicotinamide	1.3	4.4	125
" " 2	1.8	3.6	155
" " 2 + 0.5% nicotinamide	1.4	4.1	
" " 2 + 1.0% "	2.2	4.0	185
" " 2 + 2.0% "	1.5	3.7	

DISCUSSION

It is apparent from these data that large amounts of nicotinamide in the diet of rabbits and guinea pigs are innocuous. Unlike the rat, these species do not synthesize and excrete extra N^1-methylnicotinamide under these conditions. Since the effects of nicotinamide on the rat are completely alleviated by the administration of methionine, and since nicotinamide is not toxic in the diet of the rabbit or guinea pig, it seems likely that the toxicity of this compound in the diet of the rat is due solely to the synthesis of N^1-methylnicotinamide with consequent depletion of the available methionine supply. This situation, then, affords an example of a process in which an essentially innocuous substance undergoes what has been considered a "detoxication" reaction and thereby produces deficiency of a necessary dietary component.

By elucidating the mechanism of nicotinamide toxicity, the present work confirms and amplifies the conclusion earlier reached concerning the essentiality of a diet capable of supporting growth in the production of fatty livers due to choline deficiency (1, 2, 8). This conclusion has been criticized by McHenry and Patterson (9) who attributed the results obtained with nicotinamide feeding to a non-specific toxicity of unknown mechanism. The present work, however, substantiates the original hypothesis that the inclusion of nicotinamide in the rat diet is simply tantamount to feeding a methionine and, hence, choline-deficient diet. Since the objections of McHenry and Patterson appear groundless, the original conclusion remains valid. While small amounts of dietary nicotinamide permitted rat growth and induced fatty liver formation, large quantities completely inhibited growth and no fatty infiltration of the liver occurred (1, 2). The failure of fatty livers to appear in the concomitant presence of choline and thiamine deficiencies is probably, as in the case of nicotinamide feeding, an example of the general concept that the fatty livers otherwise associated with choline deficiency are not manifest when the basal diet is incapable of supporting growth.

However, it must also be stated that this conclusion applies only to short term experiments such as those which have been employed to study simultaneous choline and thiamine (10), riboflavin (11), and methionine (1) deficiencies. In experiments of longer duration[2] it has appeared that when the animals cease losing weight and the growth curves reach a plateau accumulation of fat in the liver does occur.

The author's thanks are due to Mr. Jesse W. Huff for the urinary F_2 values reported herein, and to Merck and Company, Inc., Rahway, New Jersey, for a supply of crystalline vitamins. This work was aided by a grant from the Josiah Macy, Jr., Foundation.

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[2] Handler, P., unpublished data.

A METHOD FOR THE DETERMINATION OF OXALIC ACID IN URINE

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(Received for publication, April 1, 1944)

Numerous methods have been suggested for the determination of the oxalic acid content of urine. In the early methods such as those of Salkowski (1) and Dakin (2) some combination of an ether extraction of the oxalic acid from acidified urine and a precipitation as calcium oxalate was employed. A large volume of urine was required. In an attempt to simplify the procedure Maugeri (3) introduced a method in which the ether extraction is avoided, the oxalic acid being precipitated directly as the calcium salt from only 5 cc. of buffered, albumin-free urine. A similar method was later presented by Oikawa (4) who introduced a reprecipitation of the oxalate with cerium.

Several years ago we had occasion to use Maugeri's method and experienced difficulty in obtaining checks on duplicate analyses of urine. Determinations were then made on solutions containing a known amount of oxalic acid in a salt mixture[1] made to resemble urine in its concentrations of inorganic ions. From a solution containing 50 mg. of oxalic acid per liter, which is about twice the average value for human urine, the recovery varied from 3.6 mg. to 14.8 mg. per liter. From a solution containing 100 mg. per liter the recovery was from 44.8 mg. to 62.0 mg. The fact that magnesium, phosphate, and sulfate ions affect the solubility of calcium oxalate or perhaps its rate of crystallization (5) adequately explains these unsatisfactory results. The simplicity of the method led us to a further investigation, but no satisfactory technique for direct precipitation was found.

By extracting the oxalic acid with ether from acidified urine the troublesome inorganic salts are eliminated, since they remain in the water layer. Ether extraction, however, presents problems of technique and may be incomplete (6). In the method which follows we use a modified Clausen

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[1] For convenience a solution was prepared containing 12.4 gm. of $MgSO_4 \cdot 7H_2O$, 34.8 gm. of K_2SO_4, 48.0 gm. of KH_2PO_4, and 100 gm. of NaCl per liter. A 10-fold dilution of this solution will approximate the average urinary concentration of inorganic ions. A stock solution containing 0.7443 gm. of sodium oxalate (0.500 gm. of oxalic acid) per liter in 0.01 N sulfuric acid was also prepared. To make the various solutions for analysis it was necessary only to dilute 10 cc. of the salt mixture plus the appropriate amount of the stock solution of oxalic acid to 100 cc. in a volumetric flask.

(7) apparatus by which small volumes of urine may be effectively extracted with correspondingly small volumes of ether. Bumping and other annoying features of ether extraction have been minimized. Following extraction and removal of the ether the oxalic acid is precipitated as the calcium salt from an acidified 60 per cent alcoholic solution. The oxalate is titrated with permanganate by the iodometric technique.

Apparatus

The modified Clausen continuous extraction apparatus is shown in Fig. 1. By the substitution of the "cold finger" type condenser, cork stopper connections are eliminated, thus avoiding any possible contamination from this source. Also by substituting the 300 × 25 mm. test-tube for the Kjeldahl flask smaller amounts of ether may be employed in the extraction.

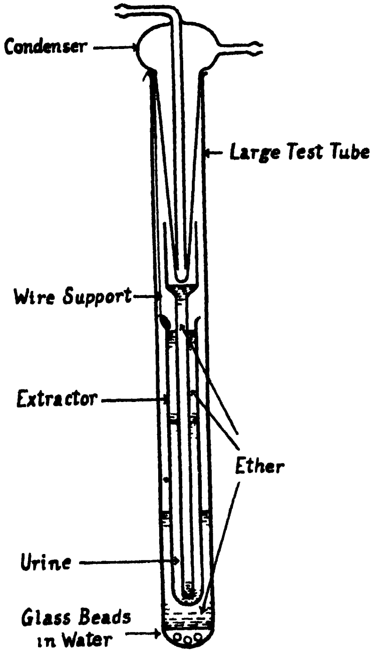

FIG. 1

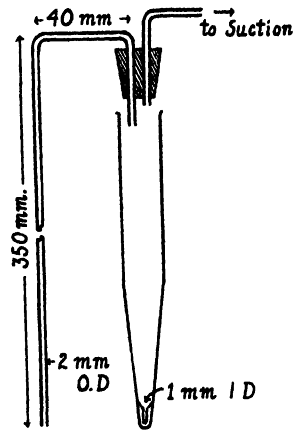

FIG. 2

FIG. 1. Extraction apparatus.
FIG. 2. Transfer siphon and centrifuge tube.

The extractor consists of a 25 cc. test-tube in which is placed a funnel-shaped tube made from half of a 25 cc. pipette. This tube has an over-all length of about 200 mm. The stem end is sealed and four holes are punched with a hot wire near the sealed end. A 3 mm. hole is drilled near the top of the test-tube and the extractor is suspended in the apparatus by a nichrome wire which is hooked through this hole and passed up over the top of the large outer tube. This also serves to raise the condenser slightly and thus provide a necessary vent.

The extraction is carried out on an electric hot-plate which should be covered with an asbestos sheet in which a small hole (about 1.5 cm. diameter) is cut. With the apparatus resting in this hole the sides are protected by the asbestos against overheating. In very hot weather a current of air from an electric fan directed against the apparatus may be necessary as an additional aid against overheating and consequent loss of ether.

The centrifuge tube shown in Fig. 2 is of 15 cc. capacity and has the tip drawn to an inside diameter of about 1 mm. This type of tube has been used by several authors (4) and is necessary in handling the very small precipitates encountered in this method. The siphon which is also shown in Fig. 2 is used to transfer the oxalic acid from the extraction apparatus to the special centrifuge tube.

Method

In the collection of the 24 hour sample of urine a few cc. of formaldehyde may be used as a preservative. Fill a 25 cc. volumetric flask to the mark with the thoroughly shaken urine. Add 1 cc. of concentrated hydrochloric acid, stopper, and mix by several inversions. Heat by immersion in boiling water for 30 minutes to hydrolyze the oxaluric acid. Cool and filter. It is then ready for extraction.

Extraction—Place three or four glass beads, 2 cc. of water, and about 25 cc. of ether in the outer tube of the extraction apparatus. This combination of glass beads and a layer of water under the ether practically eliminates the bumping which is usually such a serious problem. With a pipette transfer 10 cc. of the prepared urine to the inside tube of the apparatus which is assembled as in Fig. 1 and clamped in a support with the bottom resting on an electric hot-plate. A good flow of water must be passed through the condenser and the hot-plate so regulated that ether will drip from the condenser tip at 100 to 110 drops per minute. Continue the extraction without interruption for 6 hours. It is sometimes necessary to add more ether during the extraction by slightly raising the condenser and pouring it in from a graduate.

Transfer—Disconnect the apparatus and remove the condenser. Lift the extractor, washing it down with 2 cc. of 95 per cent alcohol from a pipette, and remove. Add 1 cc. of 2 per cent acetic acid to the contents of the outer tube. Shake with a twirling motion to mix the water and ether layers[2] and, while continuing the shaking, drive off the ether by immersion in a water bath heated to about 70°. Place the transfer siphon (Fig. 2) in the special centrifuge tube and connect with an aspirator adjusted to give a mild suction. Transfer to the special centrifuge tube is effected by inserting the long arm of the siphon to the bottom of the extraction tube. Splashing may be prevented by breaking the suction through a manipulation of the stopper as the last drops come over. Remove the siphon and wash down the sides of the extraction tube with 2 cc. of 95 per cent alcohol. Transfer this with suction to the centrifuge tube. Repeat the washing and transfer with another 2 cc. portion of alcohol. By siphoning the contents will always drain to the bottom of the tube and the transfer is easily effected.

Precipitation—Add 0.5 cc. of a 10 per cent calcium chloride solution to the contents of the special centrifuge tube and stir with an air current introduced through a fine capillary. The apparatus described by Levatinsky (9) is useful for this purpose. Overlay with 2 cc. of an acid-alcohol solution consisting of 60 cc. of 95 per cent alcohol, 10 cc. of 2 per cent acetic acid, and 20 cc. of water. This prevents the accumulation of a precipitate on the surface of the solution, which is difficult to remove by centrifuging. Let stand overnight and centrifuge for 30 minutes at about 2000 R.P.M. Decant and invert to drain for a few minutes. Wipe off the lip with a piece of filter paper. Wash down the sides of the tube with 2 cc. of the acid-alcohol solution and thoroughly break up the precipitate with a fine glass stirring rod. Remove the stirring rod, washing it down with 3 cc. of the acid-alcohol solution. Again centrifuge for 30 minutes, decant, drain, and heat for a few minutes in an oven or water bath to drive off the remaining alcohol. It is then ready for titration.

Titration—Add 1 cc. of 20 per cent sulfuric acid and 0.5 cc. of 1 per cent manganese sulfate. Break up the precipitate with a fine glass stirring rod, rinsing the rod with a few drops of water when it is removed. Add with a pipette exactly 3 cc. of 0.01 N potassium permanganate. Stir with an air current introduced through a fine capillary tube and allow to stand 8 to 10 minutes for the reaction to take place. Add 0.5 cc. of a 10 per cent solution of potassium iodide and mix by rolling between the hands. Introduce 4 drops of a 1 per cent starch solution and 2 drops of a saturated solution of barium hydroxide. (The latter is not essential, but the white precipitate of barium sulfate which is formed makes a good internal background against which one can more easily see the end-point marked by the disappearance of the blue color.) Titrate the excess permanganate with 0.01 N sodium thiosulfate with a 5 cc. micro burette graduated in 0.02 cc. while stirring with an air current. Read to the nearest 0.01 cc.

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[2] When a very dilute solution of oxalic acid in ether is evaporated to dryness, a large part of the oxalic acid is lost. This fact was noted by Khouri (8) who, to prevent the loss, added an alcoholic solution of urea to convert the oxalic acid to oxalylurea before evaporation. The treatment with urea is superfluous, however, since no loss will occur if the solution is thoroughly shaken with water during the evaporation of the ether.

Calculations—From the volume of exactly 0.01 N potassium permanganate, as determined in the back titration with thiosulfate, the reagent blank[3] is subtracted and the result multiplied by 0.45 to give the mg. of oxalic acid measured by the titration. This must be multiplied by 1.04 as a volume correction for the hydrochloric acid added. Thus if a 10 cc. sample is used, the mg. of oxalic acid in 100 cc. of urine = 4.68 $(A - B - C)$. A = 0.01 N permanganate, B = 0.01 N thiosulfate, and C = the value of $(A - B)$ found in the blank analysis.

DISCUSSION

About 90 per cent recovery of oxalic acid from known solutions is obtained by 6 hours of continuous ether extraction, as shown in Table I. These results are consistent and no advantage is to be gained by extending the extraction period to obtain complete recovery. Alcohol is employed in the transfer from the extraction tube to the special centrifuge tube because it wets the tube perfectly, thus permitting complete drainage and transfer. It also decreases the solubility of calcium oxalate in the subsequent precipitation, so that a quantitative recovery may be obtained from concentrations as low as 7.5 mg. per liter.

It might be expected that the high concentration of alcohol would result in the precipitation of the calcium salts of other organic acids such as citric, tartaric, and malic which are also present in urine and are carried over in the ether extract. Under the conditions of the precipitation in which the unbuffered solution is made distinctly acid with acetic acid the calcium salts of these acids are completely soluble. However, if oxalic acid is precipitated in the presence of citric acid, some calcium citrate is carried down. The amount precipitated consistently has a titration value equal to about 10 per cent of the calcium oxalate precipitated and is independent of the concentration of the citric acid. It is assumed to be due to occlusion

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[3] The value of this blank is so small that its origin is hard to trace. Presumably it comes mainly from the ether; yet the results are unaffected when it occasionally becomes necessary to use more ether. Doubling the amount of acetic acid likewise has no effect. Its value may be found directly by a blank analysis on distilled water. The percentage recovery by extraction as well as the reagent blank may be determined by running analyses on two known oxalate solutions, one having twice the oxalate content of the other. Thus if solutions containing 0.25 mg. and 0.50 mg. respectively are taken for analysis, titration values of 0.56 cc. and 1.06 cc. of 0.01 N permanganate may be obtained. If x is allowed to equal the blank, $2\ (0.56 - x) = 1.06 - x$. $x = 0.06$ cc. Subtracting this from the titration figures will give the values 0.50 cc. and 1.00 cc., representing a 90 per cent recovery.

TABLE I

*Recovery of Oxalic Acid from Salt Solutions**

Oxalic acid present	Oxalic acid found	
mg. per 100 cc.	*mg per 100 cc.*	*per cent recovery*
10.00	8.70	87.0
10.00	8.90	89.0
10.00	8.80	88.0
5.00	4.47	89.4
5.00	4.47	89.4
5.00	4.44	88.8
2.50	2.22	88.8
2.50	2.22	88.8
2.50	2.18	87.2
1.25	1.15	92.0
1.25	1.13	90.4
1.25	1.17	93.7
0.75	0.68	90.7
0.75	0.72	96.0
0.75	0.70	93.3
Average	.	90.2

* See foot-note 1.

TABLE II

Recovery of Oxalic Acid from Solutions Containing Citric Acid*

Each figure given is the average of duplicate analyses.

Oxalic acid present	Oxalic acid found		
	Citric acid, 0.2 gm. per liter	Citric acid, 1.3 gm. per liter	Citric acid, 6.5 gm. per liter
mg. per 100 cc.	*per cent recovery*	*per cent recovery*	*per cent recovery*
10.00		98.5	
5.00	97.0	99.2	100.0
2.50	100.8	99.0	
1.25	95.3	100.5	
0.75	97.3	99.2	

Average recovery = 98.7 per cent.

* See foot-note 1.

in the calcium oxalate precipitate. Since citric acid is a normal constituent of human urine to the amount of 0.2 gm. to 1.0 gm. per 24 hours (10) and since the amount carried down compensates within experimental error the 10 per cent loss in extraction, it is unnecessary to introduce these corrections in the calculations (Table II). The presence of the other organic acids is without effect. The presence of protein in the urine likewise presents no difficulties, as it is removed in the filtration following the water bath treatment for the hydrolysis of oxaluric acid.

The 24 hour urine samples in Table III were selected at random from subjects on average mixed diets with the exception of Sample 5. The very low oxalic acid content of this sample is a reflection of the subject's aversion to vegetables which were almost entirely lacking in his diet.

TABLE III

Oxalic Acid Excretion in 24 Hour Human Urine

Each figure given is the average of duplicate analyses.

Sample No	Excretion per 24 hrs.
	mg.
1	22.1
2	25.1
3	30.3
4	35.8
5	14.3
6	27.7
7	22.4

With known solutions of oxaluric acid it was found that heating on the water bath for a full 30 minutes was necessary for complete hydrolysis. It was hoped that the relatively low temperature of the ether extraction would have little or no hydrolytic effect on oxaluric acid and that free oxalic acid could thus be determined by a direct extraction of the unhydrolyzed urine. Unfortunately oxaluric acid is about 50 per cent hydrolyzed by this treatment. Thus the method is applicable only to the determination of total oxalic acid.

SUMMARY

A method for the estimation of oxalic acid in urine is presented which employs relatively small samples and is accurate with concentrations as low as 7.5 mg. per liter.

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STUDIES ON 1-METHYLHISTIDINE

I. A SYNTHESIS OF *dl*-1-METHYLHISTIDINE*

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(Received for publication, April 6, 1944)

1-Methylhistidine has been known since 1929 when it was obtained in inactive form on hydrolysis of anserine with barium hydroxide (Linneweh, Keil, and Hoppe-Seyler (1)). The optically active amino acid, presumably the *l*(−) isomeride, was obtained when sulfuric acid was used as the hydrolytic agent (Linneweh and Linneweh (2)).

The identification of this amino acid as 1-methylhistidine, shown below (V), was based on the following evidence. It has the elementary composition $C_7H_{11}O_2N_3$, one methylimino group, and α-amino acid character as indicated by a positive ninhydrin reaction (Linneweh, Keil, and Hoppe-Seyler (1)). On alkaline pyrolysis of anserine Linneweh and his coworkers (1) obtained a dimethylimidazole which Keil (3) and Pyman (4) later identified as the 1,5-dimethyl derivative.

1-Methylhistidine has not previously been synthesized.[1] Keil (6) obtained a mixture of 1- and 3-methyl-α-phthalylhistidines on treating α-phthalylhistidine with dimethyl sulfate, but he was unable to separate the 1-methyl from the 3-methyl derivative which he thought occurred in large excess.

A practical synthesis of 1-methylhistidine was of interest to confirm the structure of the natural compound which had been assigned that constitution and to provide a more convenient source than the laborious isolation. The synthesis of this amino acid from *d*-fructose by a series of five reactions is described below. (1) *d*-Fructose is converted into 4(5)-methylolimidazole (I). (2) 4(5)-Methylolimidazole (I) is oxidized to imidazole-4(5)-formaldehyde (II) by nitric acid. (3) Imidazole-4(5)-formaldehyde (II) is methylated to 1-methylimidazole-5-formaldehyde (III) by dimethyl sulfate in acetone. (4) 1-Methylimidazole-5-formaldehyde (III) is condensed with 2-thio-3-acetylhydantoin to 2-thio-4-(1-methylimidazole-5-al)-hydantoin (IV) with pyridine as a solvent and diethylamine or piperidine as a catalyst. (5) 2-Thio-4-(1-methylimidazole-5-al)-hydantoin (IV) is reduced and hydrolyzed in one step to form 1-methylhistidine (V) by refluxing with hydrogen iodide and red phosphorus. The yield of *dl*-1-methylhistidine is about 7 per cent based on the fructose used.

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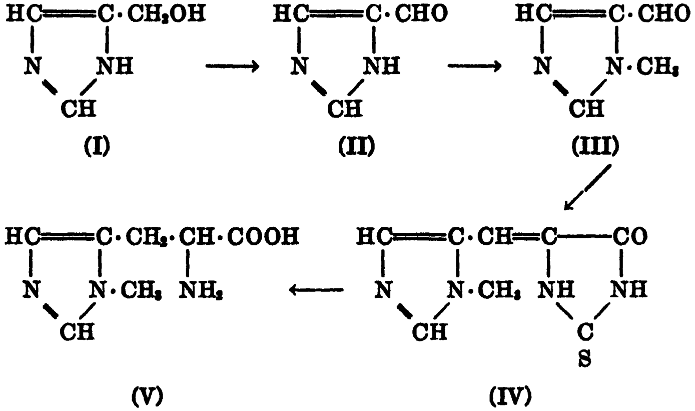
* This and the following paper are taken from the thesis presented by Warwick Sakami to the Faculty of the Graduate School of the University of Pennsylvania in 1944 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[1] The *l*-1-methylhistidine which Behrens and du Vigneaud (5) used in the synthesis of anserine was the natural compound which they obtained from Professor Ackermann.



1-Methylimidazole-5-formaldehyde picrate has been prepared from imidazole-4(5)-formaldehyde in small (5.7 per cent) yield by Hubball and Pyman (7). The procedure reported in this paper is productive of a 28 per cent yield of pure 1-methylimidazole-5-formaldehyde.

An improvement has been made in the synthesis of 2-thio-3-acetylhydantoin which was used in the preparation of 2-thio-4-(1-methylimidazole-5-al)-hydantoin. The original method (Johnson and Nicolet (8); see also Johnson (9)) in which glycine was condensed with ammonium thiocyanate in acetic anhydride was modified by the substitution of potassium thiocyanate and an equivalent of sulfuric acid for the ammonium salt. This change avoided contamination of the product with a difficultly removable colored impurity without affecting the yield. The function of the sulfuric acid is to convert the potassium thiocyanate into free thiocyanic acid; when the ammonium salt is used, this is accomplished by the acetic anhydride which acetylates the ammonia. Difference in ability to furnish thiocyanic acid is undoubtedly responsible for the unequal reactivity of ammonium and potassium thiocyanates on amino acids observed by Johnson (9) and Johnson and Nicolet (10).

dl-1-Methylhistidine was also synthesized by methods involving hippuric acid and diketopiperazine. The yields obtained by these procedures were inferior to that reported in this paper.

dl-1-Methylhistidine crystallizes from 50 per cent alcohol in microscopic rods[2] (Fig. 1). It is a sweet tasting, colorless, water-soluble compound which decomposes sharply at a temperature between 247–250°, depending on the rate of heating. Mixed melting point determinations of the 3,4-dichlorobenzenesulfonates and picrates showed that it is identical with the basic amino acid which we obtained by hydrolyzing anserine with barium hydroxide.

The 1-methylhistidine was optically inactive. A single attempt to resolve the compound by means of *d*-tartaric acid[3] was unsuccessful.

EXPERIMENTAL

4(5)-Methylolimidazole—4(5)-Methylolimidazole picrate was prepared by the procedure of Darby, Lewis, and Totter (12) in 56 per cent yield. It decomposed at 204°. The picric acid was removed by shaking with benzene and hydrochloric acid (Koessler and Hanke (13)), and the free base was isolated in 94 per cent yield essentially by the method of Pyman (14). It melted at 93–94°.

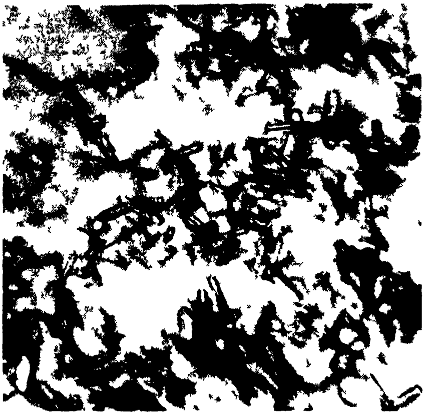


FIG 1. *dl*-1-Methylhistidine. × 150

Imidazole-4(5)-formaldehyde—4(5)-Methylolimidazole was oxidized to the aldehyde with concentrated nitric acid (Pyman (15)). The yield was 61 per cent. The product melted at 172°.

Methylation of Imidazole-4(5)-formaldehyde—20.0 gm. of imidazole-4(5)-formaldehyde were dissolved in 1600 ml. of acetone under a reflux, and the hot solution was filtered from a small amount of resin. 20.0 ml. of freshly distilled dimethyl sulfate were added drop by drop while the flask was shaken, and the solution was heated under a reflux for 1 hour. The acetone was removed under reduced pressure, leaving an amber-colored oily liquid which was dissolved in 30 ml. of water and extracted with a little chloroform which was discarded. The aqueous solution was saturated with potassium carbonate and extracted with 500 ml. of chloroform in 50 ml. portions. After the chloroform extract was dried with anhydrous magnesium sulfate, it was evaporated under reduced pressure. The residue set to a mass of yellow crystals weighing 10.6 gm.

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[2] Crystals resembling those shown in Linneweh and Linneweh's (2) photograph were obtained on evaporating a drop of aqueous *dl*-1-methylhistidine solution on a watch-glass.

[3] *dl*-Histidine has been resolved with *d*- and *l*-tartaric acids (Pyman (11)).

1-Methylimidazole-5-formaldehyde Monopicrate—The mixture of methylimidazoleformaldehydes was dissolved in 100 ml. of water and poured into a hot solution of 22.1 gm. (1 equivalent) of picric acid in 800 ml. of water. The solution was rapidly cooled to room temperature in a water bath and filtered. The yellow crystalline precipitate was washed with a little water and dried *in vacuo* over sulfuric acid. The yield, 20.1 gm., was 28 per cent of the theory based on the imidazole-4(5)-formaldehyde used. The picrate melted at 172–173°, simultaneously with an authentic sample of 1-methylimidazole-5-formaldehyde picrate prepared according to the directions of Hubball and Pyman (7) and with a mixture of the two preparations. A sample was prepared for analysis by recrystallization from water. The melting point was not improved by this procedure.

Analysis (*Kjeldahl*)—$C_5H_6ON_2 \cdot C_6H_3O_7N_3$. Calculated, N 20.6; found, 21.0

1-Methylimidazole-5-formaldehyde—20.0 gm. of 1-methylimidazole-5-formaldehyde picrate were shaken with a mixture of 40 ml. of 12 per cent hydrochloric acid and 80 ml. of nitrobenzene. The nitrobenzene was separated, and the remaining picric acid and nitrobenzene were carefully removed from the aqueous solution by extracting three times with 10 ml. portions of chloroform. The solution was then saturated with potassium carbonate and extracted with 600 ml. of chloroform in 50 ml. portions. The chloroform was dried with anhydrous magnesium sulfate and evaporated under reduced pressure. 6.45 gm. of colorless crystals melting at 54° were obtained. This is a yield of 99.5 per cent. The material was analyzed without further purification.

Analysis (*Kjeldahl*)—$C_5H_6ON_2$. Calculated, N 25.5; found, 25.0

2-Thio-3-acetylhydantoin—10.0 gm. of glycine and 12.9 gm. of potassium thiocyanate were intimately ground together and suspended in 45 ml. of acetic anhydride and 5 ml. of glacial acetic acid. Reaction occurred spontaneously in a few minutes. When the solid had dissolved, 3.6 ml. of concentrated (95 to 96 per cent) sulfuric acid (Merck reagent) were added drop by drop while the flask was shaken, and the solution was then heated on the steam bath for 20 minutes. The product was stirred into 165 ml. of ice-cold water and placed in the refrigerator. After the mixture stood 4 hours, the straw-colored crystals were filtered off, washed with water, and dried over sulfuric acid. 15.0 gm. of 2-thio-3-acetylhydantoin were obtained, which softened at 171° and melted at 173–174°. After recrystallization of the product from 100 ml. of alcohol it weighed 11.5 gm., melted at 175°, and was colorless. The yield of purified product was 55 per cent. The compound did not depress the melting point of an authentic sample of 2-thio-3-acetylhydantoin prepared according to the directions of Johnson and Nicolet (8).

2-Thio-4-(1-methylimidazole-5-al)-hydantoin—1.00 gm. of 1-methylimidazole-5-formaldehyde and 1.44 gm. of 2-thio-3-acetylhydantoin[4] were suspended in 2 ml. of pyridine. On addition of 2 ml. of diethylamine[5] a vigorous reaction commenced and sudden precipitation occurred after a few minutes. The mixture was then heated under a reflux on a water bath for 10 minutes and poured into 100 ml. of hot[6] water. 2 ml. of glacial acetic acid were added immediately, and the precipitate was filtered after cooling to room temperature. The product was thoroughly washed with water and dried, first over sulfuric acid, then by heating 1 hour at 100° *in vacuo* over phosphorus pentoxide. The yield was 1.79 gm., 95 per cent of the theory. It decomposed at 321°. A sample purified for analysis by dissolving in dilute hydrochloric acid and precipitating with dilute sodium hydroxide decomposed at 333°.

Analysis (*Kjeldahl*)—$C_8H_8OSN_4$. Calculated, N 26.9; found, 26.5

dl-1-Methylhistidine Bis-3,4-dichlorobenzenesulfonate—1.00 gm. of the thiohydantoin was refluxed 6 hours with 10 ml. of constant boiling hydriodic acid and 0.5 gm. of red phosphorus. The hydriodic acid was then removed *in vacuo* and the residue taken up in a little water and filtered from excess phosphorus. The final volume of the solution was 35 ml. 3.5 gm. of 3,4-dichlorobenzenesulfonic acid[7] were stirred in rapidly. A dense crystalline precipitate (Fig. 2) separated almost immediately and was filtered off after standing in the refrigerator for 2 hours. The product was washed with a small volume of ice-cold 5 per cent aqueous 3,4-dichlorobenzenesulfonic acid solution, dried over sulfuric acid, and freed from traces of the sulfonic acid by washing with ether. It weighed 2.72 gm. and melted at 251–252°. The yield was 91 per cent. A sample was prepared for analysis by recrystallizing it from water. This procedure did not raise the melting point.

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[4] The use of a slight excess of acetylthiohydantoin does not increase the yield.

[5] Piperidine may be used with identical results.

[6] The thiohydantoin is readily filtered only when it is precipitated from hot solution.

[7] 3,4-Dichlorobenzenesulfonic acid was prepared by Vickery's procedure (16).

Analysis (*Kjeldahl*)—$C_7H_{11}O_2N_3 \cdot 2C_6H_4O_3SCl_2$. Calculated. N 6.74
Found. " 6.77

dl-1-Methylhistidine—A solution of 9.5 gm. of barium hydroxide dissolved in 25 ml. of hot water was added to a hot solution of 15 gm. of *dl*-1-methylhistidine bis-3,4-dichlorobenzenesulfonate in 75 ml. of water. After the mixture was chilled in the refrigerator 2 hours, the barium 3,4-dichlorobenzenesulfonate was filtered off and thoroughly washed with cold water. The filtrate and washings were combined and concentrated to about 25 ml. After the material had stood in the refrigerator ½ hour, a small amount of the barium sulfonate was filtered off and carefully washed with a little ice-cold water which was combined with the filtrate. The solution was freed from barium with a slight excess of sulfuric acid, and the filtrate concentrated to about 10 ml. and precipitated with an equal volume of alcohol. After the product was cooled in the refrigerator ½ hour, the crystals were filtered off, washed with a little 50 per cent alcohol, then with a few drops of 95 per cent alcohol, and sucked dry on the funnel. 3.36 gm. of *dl*-1-methylhistidine were obtained. The compound decomposed sharply at a temperature between 247–250°, depending on the rate of heating.

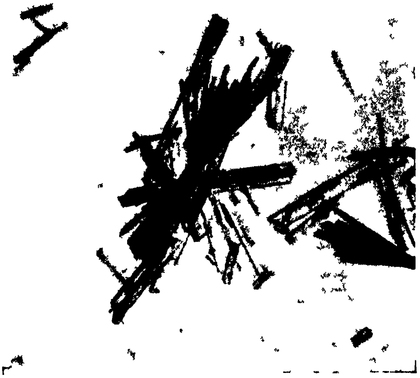

FIG. 2. *dl*-1-Methylhistidine bis-3,4-dichlorobenzenesulfonate × 35

A second crop of *dl*-1-methylhistidine, weighing 0.39 gm., was obtained on concentrating the filtrate to 2 ml. and precipitating with an equal amount of alcohol. Its decomposition point was 2° below that of the first crop. On recrystallization by dissolving in 1.5 ml. of hot water and precipitating with 1 ml. of alcohol, 0.35 gm. of *dl*-1-methylhistidine was obtained, which melted identically with the first crop.

The total yield of pure *dl*-1-methylhistidine was 3.71 gm., 91.2 per cent of the theory. The material was analyzed without further purification.

Analysis—$C_7H_{11}O_2N_3$
Calculated. C 49.69, H 6.56, N 24.8, N-Me 8.88
Found. " 49.64, " 6.58, " (Kjeldahl) 24.6, N-Me 8.93

The Beilstein test for halogen was negative.

1.27 gm., 8.5 per cent, of the *dl*-1-methylhistidine bis-3,4-dichlorobenzenesulfonate were recovered by evaporating the combined filtrates from the precipitation and recrystallization of the *dl*-1-methylhistidine to about 2 ml. and adding 1.25 gm. of the sulfonic acid.

The properties of *dl*-1-methylhistidine agree with those reported in the literature. It gives a positive ninhydrin reaction and a positive Kapeller-Adler test though with a weaker color than histidine. Its aqueous solutions dissolve cupric carbonate on warming with the formation of a blue color.

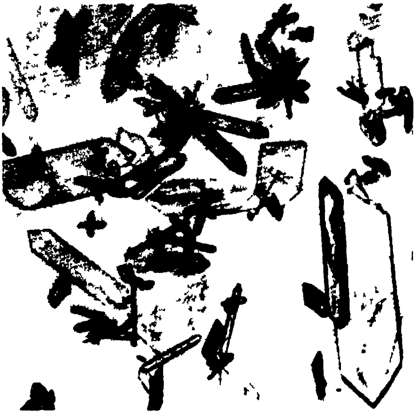

FIG. 3. *dl*-1-Methylhistidine dipicrate. × 35

dl-1-Methylhistidine Dipicrate—A dipicrate of *dl*-1-methylhistidine was prepared by adding an excess of saturated aqueous picric acid to a concentrated solution of the amino acid. It precipitated as an oily liquid which solidified on scratching. After recrystallization from water (yellow prisms, Fig. 3) and drying over sulfuric acid, it decomposed at 186°.

Analysis (Kjeldahl)—$C_7H_{11}O_2N_3 \cdot 2C_6H_3O_7N_3$. Calculated, N 20.1; found, 20.0

Identification of dl-1-Methylhistidine with Basic Amino Acid Obtained on Hydrolyzing Anserine with Barium Hydroxide—Anserine was hydrolyzed by a modification of the procedure of Linneweh, Keil, and Hoppe-Seyler (1). 0.20 gm. was refluxed for 24 hours with 5 gm. of barium hydroxide and 5 ml. of water. After the solution was diluted, most of the barium was removed as the carbonate and the remainder was precipitated by adding a slight excess of sulfuric acid. A polarimetric analysis indicated that the 1-methylhistidine was 96 per cent racemized. *dl*-1-Methylhistidine was isolated as the 3,4-dichlorobenzenesulfonate and recrystallized from water. The appearance of the crystals was indistinguishable from that of synthetic *dl*-1-methylhistidine bis-3,4-dichlorobenzenesulfonate. The melting point of the compound, 251–252°, was not depressed on mixture with the synthetic preparation.

Analysis (*Kjeldahl*)—$C_7H_{11}O_2N_3 \cdot 2C_6H_4O_3SCl_2$. Calculated. N 6.74
Found. " 6.65

The 3,4-dichlorobenzenesulfonate was converted into the picrate and recrystallized from water. The compound was dried over sulfuric acid, then over phosphorus pentoxide *in vacuo* at 100°. The crystals had the same appearance as that of synthetic *dl*-1-methylhistidine dipicrate. The decomposition point, 185°, was not depressed by mixture with the synthetic compound.

Analysis (*Kjeldahl*)—$C_7H_{11}O_2N_3 \cdot 2C_6H_3O_7N_3$. Calculated, N 20.1; found, 19.8

SUMMARY

dl-1-Methylhistidine has been synthesized. It is identical with the basic amino acid obtained on hydrolyzing anserine with barium hydroxide.

An improved synthesis of 2-thio-3-acetylhydantoin has been described.

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STUDIES ON 1-METHYLHISTIDINE

II. A STUDY OF THE METABOLISM OF *dl*-1-METHYLHISTIDINE IN THE ALBINO RAT

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(Received for publication, April 6, 1944)

1-Methylhistidine is a component of anserine, a normal constituent of the muscles of many vertebrates (Zapp and Wilson (1)). In a recent communication (Schenck, Simmonds, Cohn, Stevens, and du Vigneaud (2)) its formation in the rabbit by the transfer of methyl groups from methionine to histidine has been described. A study of the possibility that the methyl group can be removed is discussed in the present paper. Processes of this type have been demonstrated for the methylglycines; *i.e.*, sarcosine, N-dimethylglycine, and betaine. When sarcosine (Bloch and Schoenheimer (3)) or betaine (Stetten (4)) containing an excess of N^{15} was fed to adult rats, the glycine isolated from the tissue proteins was found to contain the isotope in much higher concentration than other amino acids, indicating that these compounds can be converted into glycine by demethylation without deamination. Handler and his coworkers (5) have observed processes of this nature in "broken cell preparations" of rat, rabbit, and guinea pig liver in that added sarcosine and N-dimethylglycine were oxidized to glycine and formaldehyde.

Since histidine is an essential amino acid for the albino rat, it is possible to test the hypothesis that these animals may obtain histidine from *dl*-1-methylhistidine[1] by feeding this compound as a supplement to a histidine-deficient diet. This experiment has been carried out as described below.

EXPERIMENTAL

Five litter mate albino rats 26 days old were confined in separate cages and fed *ad libitum* a diet of the following percentage composition: starch 40.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Jones and Foster (8)) 4.0, agar 2.0, histidine-deficient casein hydrolysate[2] 14.5, tryptophane 0.2, and cystine 0.3. Vitamin B complex was provided by the separate daily feeding of 100 mg. of yeast vitamin (Harris).

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[1] *d*-Histidine supports the growth of rats nearly as well as *l*-histidine (Cox and Berg (6), Conrad and Berg (7)).

[2] The histidine-deficient casein hydrolysate was prepared by the procedure of Conrad and Berg (7). Casein was hydrolysed with sulfuric acid and the histidine was precipitated with mercuric sulfate.

The weight and food consumption were measured at frequent intervals and are summarized in Fig. 1. On replacement of the laboratory stock diet with the basal food mixture deficient in histidine, the rats at first lost weight rapidly. By the 9th day their weights were approximately constant, and the administration of supplemented diets was commenced. Rat 1 was given a supplement of 0.37 per cent *l*-histidine and Rats 2, 3, and 4 supplements of 0.80 per cent *dl*-1-methylhistidine in the basal diet. The *dl*-1-methylhistidine was prepared by the procedure reported in Paper I (Sakami and Wilson (9)). Rat 5 was retained as a control. The animal receiving histidine gained weight and increased its food consumption, while the others all continued to decline in weight. After the 22nd day all rats except the control were fed the basal diet supplemented with 0.37 per cent *l*-histidine. Rat 4 received in addition 0.80 per cent *dl*-1-methylhistidine. They all consumed more food and increased in weight.

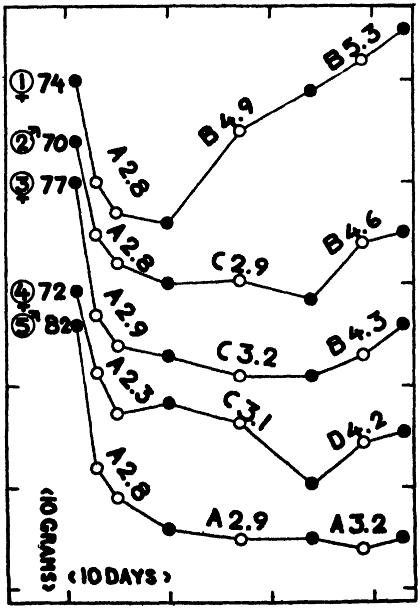

FIG. 1. Growth of albino rats on a basal diet deficient in histidine and on this diet supplemented with *l*-histidine and *dl*-1-methylhistidine. Sex and initial weights of the rats are indicated to the left of the curves. The figure within the sex symbol is the number of the rat. Letters and figures above the curve denote the diet and average food consumption per day respectively, computed for the period between the solid circles. Diet A is the basal diet; Diet B is Diet A + 0.37 per cent *l*-histidine; Diet C is Diet A + 0.80 per cent *dl*-1-methylhistidine; Diet D is Diet A + 0.37 per cent *l*-histidine + 0.80 per cent *dl*-1-methylhistidine.

DISCUSSION

When compared with respect to food consumption and loss in weight, the three albino rats which received a food mixture deficient in histidine supplemented with 0.80 per cent *dl*-1-methylhistidine were similar to an animal which was given only the basal diet, and to Conrad and Berg's (7) rats which received a closely similar basal food mixture. This supplement was sufficient to supply 0.37 per cent *d*- or *l*-histidine if demethylation of one of the isomers occurred. On the other hand, when the *dl*-1-methylhistidine was replaced with 0.37 per cent *l*-histidine the rats consumed more food and increased in weight. It is evident that under these experimental conditions the albino rat cannot obtain histidine from either the *d*- or the *l*-1-methyl derivative. Our experiment does not indicate whether this amino acid can furnish methyl groups for the methylation of other compounds, but if this process occurs, the product is not histidine and cannot be converted into it.

It has been reported that anserine is little if at all toxic (Isachenko and Filippova (10)). No evidence was obtained in this experiment that *dl*-1-methylhistidine is toxic for the white rat. The rapid loss in weight of Rat 4 was probably due to the histidine deficiency, since this animal gained weight when it received *l*-histidine even though the methyl derivative was retained in its diet.

SUMMARY

dl-1-Methylhistidine did not promote growth in albino rats when fed as a supplement to a diet deficient in histidine. This shows that these animals cannot obtain histidine by the demethylation of this derivative.

Dietary *dl*-1-methylhistidine appears to be well tolerated by albino rats when fed at a level of 0.80 per cent.

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THE ACTION OF 1,2-EPOXIDES ON PROTEINS

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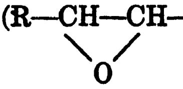
(Received for publication, February 28, 1944)

During a study of methods for the modification of waste proteins for industrial use, the scarcity of reagents known to combine with protein carboxyl groups became evident. Indications that the carboxyl groups of silk fibroin and of wool might be esterified by treatment with diazomethane (1) or methyl sulfate or halide (2) have been reported. The conditions of the usual methods for esterification, however, may be harmful for many proteins (3). It appeared that 1,2-epoxides  $(\mathrm{R-\underset{\diagdown\,O\,\diagup}{CH-CH}-R'})$ might prove practicable, inasmuch as they are known to combine with acids under mild conditions (4). Several representatives of this class of compounds, such as ethylene oxide, 1,2-propylene oxide, and epichlorohydrin, are now commercially available. A search of the literature revealed no information concerning the nature of the interaction of these reagents with proteins, other than a patented procedure for the esterification of casein (5). The action of epoxides on amino acids appears to have been studied exclusively in anhydrous media (6, 7). Model experiments on the esterification of fatty acids and amino acids by epoxides in aqueous solution at room temperature were therefore initiated simultaneously with an investigation of the effect of these reagents on several proteins. Some results of the former experiments are being reported elsewhere.[1] They indicated that epoxides were effective esterifying agents for dissociated carboxyl groups. Amino groups, on the other hand, were found to combine with the reagent more rapidly when uncharged.

Treatment with epoxides will be shown here to be similarly effective for modifying proteins in aqueous solution at room temperature. While this publication will be confined to the studies performed on crystalline egg albumin and β-lactoglobulin, other proteins were found to react generally in a similar manner.[1] The reactions which were found to occur can be illustrated by the accompanying scheme.

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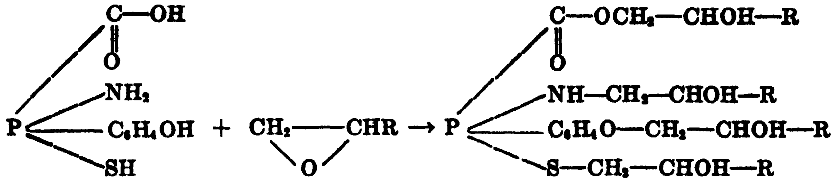
* This is one of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

[1] Fraenkel-Conrat, H., Olcott, H. S., and Cooper, M., in preparation for publication.



The most pronounced effect on the properties of the protein is due to the esterification of a great proportion of its carboxyl groups. This causes a shift in the isoelectric point by 1 to 3 pH units. The solubility of the protein in neutral and alkaline solution is diminished and the electrophoretic mobility is affected.

The addition of the reagent to the amino groups does not suppress their basic nature and therefore does not tend to balance the effects of esterification of carboxyl groups on the properties of the protein.

Phenolic and sulfhydryl groups form ethers and thio ethers with the epoxide reagents. A study of the reaction of aliphatic hydroxyl groups is precluded by the lack of a test for these groups in proteins, but it may be assumed that aliphatic ethers do not form under the experimental conditions used, since the introduction of many new hydroxyl groups by combination of the reagent with all types of protein groups would then be expected to lead to unlimited polymerization. Actually the yield of the protein derivative is limited, the amount of reagent introduced never exceeding 10 per cent of the weight of the protein, regardless of the excess used.

EXPERIMENTAL[2]

Preparation of Derivatives—The reaction between epoxides and proteins was performed under various conditions, four of which offered definite and specific advantages. These were (1) reaction in neutral salt-free solution, (2) reaction in solution acidified with acetic acid to pH 3.5 (approximately 0.4 gm. of acid per gm. of protein),[3] (3) reaction in sodium hydroxide solution of pH 8 (approximately 2.7 ml. of 0.1 N alkali per gm. of protein), and (4) reaction in 6.6 M urea. The egg albumin samples used in the experiments were placed at our disposal by F. E. Lindquist and R. A. O'Connell of this Laboratory; they were prepared according to Kekwick and Cannan (8) and were recrystallized from two to four times. The crystalline β-lactoglobulin was kindly supplied by E. F. Jansen of this Laboratory who prepared it according to the method of Palmer (9). Ethylene and propylene oxides were commercial preparations; epichlorohydrin was furnished by the Shell Development Company.

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[2] The details of representative experiments are summarized in Table I.

[3] Hydrochloric acid was found unsuitable since it combined more rapidly with the reagent than did the protein.

TABLE I

Effect of Epoxides on Proteins; Experimental Details

Method No.	Conditions of treatment				Reaction product						
	Concentrations of			Time	Solubility	Yield	Total N	Amino N†	Fraction of original†		
	Protein	Epoxide*	Other reagents						Phenol (+ indole)	Acid groups	Basic groups
	per cent	*per cent*	*per cent*	*days*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
	Egg albumin										
1	3.3	13		1	Ppt.	100	14.2	0.22	63	54	
	1.46	17		4	“	107	13.4	0.15	36	26	109
	1.64	6.4		2	“	93	14.5	0.21	<43‡	49	
	1.67	4.8*		2	“	94	14.3	0.17	<56‡	38	
2	2.05	14	Acetic acid, 0.75 (pH 3.5)	2	Soluble	90	14.7	0.60	<100‡	65	137
	1.52	13	Acetic acid, 0.61	6	“	92	14.6	0.44	<56‡	52	
3	3.0	12	NaOH, 0.04 (pH 8.1)	1	Gel	101	13.9	0.03	64	55	
	0.93	13	NaOH, 0.01	2	Soluble	107	13.5	0.05	27	26	106
	2.2	9.5	“ 0.03	7	“	112	13.6	0.03	27	18	112
	1.67	14.3	NaCl, 0.08	2	Ppt.-gel	116	13.7	0.05	50	31	
4	2.1	8.3	Urea, 40	1	Soluble	96	13.9			60	
	0.93	13	“ 40	2	“	107	13.7	0.06	<40‡	30	100
	1.19	13	“ 40	4	“	120	13.8	0.03	<28‡	20	
	1.19	13	“ 40	7	“	110	13.7	0.03	20	22	
	β-Lactoglobulin										
8	6.4	20	NaCl, 0.8	1	Ppt.-gel		12.9	0.12	76	44	
	3.1	14	“ 0.4	2	Soluble	91	12.9			37	
	6.7	17	“ 0.8	4	Ppt.-gel	88	12.7	0.18	48	29	111
	Untreated egg albumin						15.0	0.63	100	100	100
	Untreated β-lactoglobulin						15.0	1.25	100	100	100

* Propylene oxide was used in all experiments except that marked with an asterisk in which ethylene oxide was used at a concentration equivalent to that of propylene oxide in the preceding experiment.

† None of these data is corrected for the increase in mass of the protein through combination with the reagent.

‡ These results were obtained from readings of turbid solutions.

More work was done with propylene oxide than with the lower boiling ethylene oxide or the water-insoluble epichlorohydrin. A comparison of the properties of egg albumin derivatives prepared by the use of equimolar amounts of the three reagents indicated no major difference, with the exception that the epichlorohydrin-treated material was less soluble than the other two.

When an epoxide was added to a dialyzed egg albumin solution (1.5 to 4 per cent), a turbidity appeared within a few seconds, followed by gradual precipitation of the protein (Method 1). Complete precipitation of the derivative occurred when the reagent concentration was 10 to 20 per cent; only 30 to 50 per cent of the protein was precipitated at epoxide concentrations of 3 and 5 per cent. The insoluble protein derivative could be isolated by centrifugation. Repeated washing with 0.1 M sodium chloride solution did not cause losses of the material exceeding 5 per cent.

When the treatment was carried out in acid or alkaline solution, or in urea (Methods 2, 3, 4), no precipitate formed even upon prolonged standing. The alkaline solutions were slowly transformed into clear gels if the protein concentration was high (3 to 4 per cent). When the protein was treated with propylene oxide in 0.5 to 1.0 per cent sodium chloride solution, a precipitate formed and again disappeared within about 16 hours, resulting in a clear solution or gel, depending upon protein concentration. This phenomenon could be explained as due to the known tendency of epoxides to add hydrochloric acid, even from neutral solutions (4), with the formation of free hydroxyl ions. Propylene oxide treatment in salt solution therefore corresponded to that performed with added alkali (Method 3), as was borne out by the physical and chemical properties of the resulting protein derivatives.

Regardless of the nature of the solvent, the reaction of proteins with epoxides was found to be accompanied by a shift in the pH of the reaction mixture toward the alkaline side. This shift had previously been observed also with amino acids[1] and is a direct consequence of the esterification of carboxyl groups.

To isolate the reaction products, the solutions (or gels) were dialyzed; this increased the tendency toward gel formation. Electrodialysis was then found convenient, leading to precipitation of the entire modified protein, regardless of its previous physical state. The salt-free suspensions could be centrifuged satisfactorily, notwithstanding the gelatinous appearance of the electrodialyzed protein. The precipitates were frozen and dried *in vacuo*, yielding the modified protein in the form of a light powder, generally in yields of 100 to 110 per cent. While the preparation of the protein derivatives by these methods was slightly more laborious than by precipitation from neutral solution (Method 1), it offered definite advantages. Thus treatment of the protein in alkali or in urea favored the reaction of the amino groups with the reagent. Variations in the conditions of treatment also affected the solubility of the protein derivative, as will be demonstrated in the following section.

Solubility of Derivatives—As indicated by the methods of isolation, all protein derivatives were almost completely insoluble in distilled water and in dilute salt solutions. They were, however, soluble in 40 per cent urea. Their solubility in acid and alkaline solutions corresponded to what might be expected of protein derivatives containing fewer acid groups but the original number of basic groups. Thus all derivatives studied were soluble in dilute acid, but much less soluble or insoluble in alkali.

Preparations obtained by Method 1 were the least soluble. They could be dissolved only at approximately pH 3, or at pH 2.5 if epichlorohydrin had been used in their preparation. Subsequent addition of alkali gave rise to a turbidity followed by precipitation when the pH rose to 4.5 to 6.5, depending upon protein concentration. Addition of alkali up to pH 11 or 12 generally resulted in resolution of the material, but the product could not be dissolved by the same amount of alkali directly; *i.e.*, without previously having been dissolved in acid. An explanation for this was suggested by the finding that a small part of the ester linkages was not stable in acid solutions, as will be shown below.

The derivatives prepared in acid or alkali (Methods 2 and 3) were more soluble than those obtained by Method 1. They could be dissolved directly in alkali as well as in a weakly acidic medium (at pH 11 or 6, to 1 per cent or more). The same was the case for preparations obtained by Method 4 if treatment was restricted to a period of 1 or 2 days only. After 4 days treatment in urea, the product was considerably less soluble.

Isoelectric Point of Derivatives—The fact, already mentioned, that epoxide-treated egg albumin and β-lactoglobulin preparations were least soluble between pH 7 and 8 suggested a shift of their isoelectric points by about 2 to 3 pH units. Owing to their insolubility in the isoelectric region, indirect methods were used in search of confirming evidence. Electrodialyzed preparations of both treated (Method 4, 4 days) and untreated egg albumin were dissolved in 6.6 M urea (to 1.5 per cent) and the pH values of these solutions were determined by means of a glass electrode. These were pH 5.7 for the untreated and pH 8.7 for the treated sample. While both these values were slightly higher than expected, possibly owing to an error in the pH measurements introduced by the solvent (40 per cent urea), the difference may be regarded as further evidence for a shift of the isoelectric point of the treated protein.

Confirmation was also obtained from studies of the dye-combining capacity of the proteins and their derivatives. Chapman, Greenberg, and Schmidt (10, 11) showed that negatively charged protein groups combined with basic dyes and positively charged groups with acid dyes. On the basis of the assumption that the isoelectric point of a protein corresponds to the pH at which the capacity of the protein to combine with both acid and basic dyes is at a minimum, the use of dyes has been suggested for the approximate determination of the isoelectric point of soluble and insoluble proteins.[1] It was observed that the esterified derivatives of both egg albumin and β-lactoglobulin bound no measurable amounts of the acid or basic dyes between pH 6 and 8, while the untreated proteins bound no dye at pH 5.

Further confirmation of the change in the isoelectric point of the treated proteins came from electrophoretic experiments.

Electrophoretic Migration—These experiments were kindly performed by Dr. H. P. Lundgren and Dr. W. Ward of this Laboratory by the conventional Tiselius technique. The results of representative runs, summarized in Table II, show that propylene oxide-treated egg albumin and β-lactoglobulin migrated toward the cathode at pH 5.8 and 6.0, while the untreated proteins migrated anodically at this reaction. At pH 9.6 to 9.9 all samples migrated toward the anode, the treated ones, as might be expected, at a lower rate than the untreated preparations. With regard to homogeneity, the epoxide derivatives included in Table II appeared to be of the same order of "purity" as the untreated proteins. The latter may have contained as much as 20 per cent of a contaminant of similar mobility as the main fraction, as indicated by a single but not quite symmetrical boundary. In contrast to these more soluble epoxide derivatives obtained by Methods 2, 3, and 4, most of those prepared according to Method 1 had to be run in the Tiselius apparatus below pH 4, owing to their insolubility in salt solutions of higher pH. These latter preparations showed inhomogeneity to a considerable extent, due perhaps to the method

TABLE II

Effect of Propylene Oxide Treatment on Electrophoretic Mobility of Proteins

Protein and type of treatment	Electrophoretic mobility*	pH†
	sq. cm. per volt sec. × 10^5	
Egg albumin, untreated....................	−4.4	5.8
" " Method 1, 1 day.............	+3.0	5.5
" " " 2, 7 days.............	+5.1	5.8
" " untreated...................	−8.8	9.5
" " Method 4, 1 day..........	−6.2	9.6
" " " 3, 7 days.....	−4.1	9.5
β-Lactoglobulin, untreated........	−2.9	5.9
" Method 3, 1 day.......	+0.73	6.0

* Descending boundary.

† Acetate buffers were used at pH 5.5 to 6.0; a glycine buffer at pH 9.5 to 9.6. The ionic strength was 0.03 to 0.04 throughout.

of preparation or to an action of the buffers of pH 2.5 and 3.5 on the protein derivatives.

Determination of Protein Groups

Amino, Phenolic, and Sulfhydryl Groups—Amino groups were estimated by the Van Slyke manometric procedure (12), with a 15 minute reaction period in a chamber protected from light (13). The amino nitrogen content of various protein preparations was found to be largely independent of the physical state of the material; thus, similar values were obtained for suspensions of 60 to 200 mesh powders and for acid solutions of the same material. Amino nitrogen determinations of various epoxide-treated preparations indicated that the amino groups of proteins had reacted almost quantitatively when the treatment was performed in alkali or in urea (Methods 3 and 4). The derivatives prepared by rapid precipitation from neutral solution (Method 1) still retained about 30 to 40 per cent, and those prepared in acid (Method 2) 70 to 100 per cent, of their original amino groups (Table III). These findings are in agreement with those of model experiments on amino acids,[1] which indicated that the amino groups reacted preferentially in alkaline solution.

TABLE III

Effect of Propylene Oxide Treatment on Protein Groups

	Method No.	Conditions of treatment		Decrease in groups*		
		Medium	Days	Carboxyl	Amino	Phenol (+ indole)
				per cent	*per cent*	*per cent*
Egg albumin + propylene oxide	1	Neutral	1	44	63	34
			2	49	66	55
			4	70	73	60
	2	Acid	2	34	2	>0
			6	46	27	>42
	3	Alkali	1	41	95	31
			2	68	89	66
			7	78	95	70
		Saline	2	59	91	50
	4	Urea	2	67	87	>55
			4	78	95	>69
			7	76	95	78
Egg albumin + ethylene oxide	1	Neutral	2	60	71	41
	4	Urea	2	77	85	80
β-Lactoglobulin + propylene oxide	3	Saline	1	49	88	12
			4	66	72	43

* Corrected for the increase in mass of the protein through combination with the reagent, as indicated by decreases in nitrogen content (see Table I).

Other protein groups for which test methods were available were the phenol (plus indole) groups and the sulfhydryl groups. For these colorimetric tests, the protein had to be used in solution rather than in suspension. The Herriott modification (14) of the Folin method was employed for the estimation of the phenolic groups. The readings obtained with protein derivatives were compared with a standardization curve prepared with 2 to 20 mg. of native egg albumin.[4] Upon addition of the reagent and buffer, some of the samples studied yielded turbid solutions which could not be clarified by centrifugation. No attempts were made to avoid this by the use of a solvent, such as urea or a detergent, which would have introduced the unknown factor of the effect of denaturation through the solvent on the available tyrosine groups (15). Clear solutions were obtained with a sufficient number of samples to permit the conclusion that the phenolic groups had been blocked to about 80 per cent by both propylene oxide and ethylene oxide in urea solution, slightly less in alkaline and neutral solution without urea, and least in acid solution (Table III).

Sulfhydryl tests with the Folin reagent in urea solution (16) were negative for the derivatives of both egg albumin and β-lactoglobulin. Thus it appeared that the original protein thiol groups had been transformed into thio ethers.

Acid and Total Basic Groups—While the apparent changes in solubility, isoelectric point, and electrophoretic behavior of the treated proteins strongly suggested that the carboxyl groups had been esterified, further proof and in particular a quantitative measure of this reaction had as yet to be found. Titration curves were determined repeatedly, but, owing to the extended range of insolubility of the modified proteins, these curves were necessarily of only limited significance.

The finding of Chapman, Greenberg, and Schmidt (10, 11) that acid and basic dyes combined stoichiometrically with basic and acid protein groups supplied a convenient tool for the estimation of these groups. An application of this finding to a procedure suitable for routine analysis is described in the following paper. With this method the number of acid groups of proteins was found markedly decreased by treatment with epoxides, indicating the esterification of 45 to 78 per cent of the original carboxyl groups. Treatment for several days in alkaline or urea solution (Methods 3 and 4) led to the disappearance of a slightly larger proportion of the carboxyl groups than treatment in neutral solution (Method 1); the acid medium (Method 2) was least favorable for esterification. Ethylene oxide was about as effective as propylene oxide in decreasing protein carboxyl groups.

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[4] This curve was not a straight line, in contrast to that for free tyrosine from 0.15 to 0.5 mg.

Determinations by dye methods of the total basic groups of various propylene oxide-treated protein preparations yielded results which were similar to or higher than those obtained with the untreated proteins (Table I).[5] Thus conversion of the primary amino groups to secondary and possibly tertiary isopropanol amines did not affect their basic nature sufficiently to prevent them from combining with an acid dye at pH 2.2.

Elementary Analyses of Derivatives—In view of the finding that epoxides reacted with a great number of protein groups, it was thought that the consequent depression in the nitrogen content of the protein derivatives might represent a quantitative measure of the over-all extent of interaction. For this purpose electrodialyzed samples were analyzed by the Kjeldahl method. The nitrogen content of untreated egg albumin was found to be 15.0 per cent. That of treated preparations ranged down to 13.4 per cent (Table I).[6] These data indicated that the amount of epoxides combined with egg albumin represented as much as 9 per cent of the weight of the derivative. From this it can be calculated that 80 moles of propylene oxide may be bound by 1 mole of egg albumin, which corresponds to the number of protein groups found blocked by analyses, namely four-fifths of the carboxyl and phenolic groups (forty-one and eight respectively) and all of the amino and sulfhydryl groups (twenty-two and seven respectively).[7] It must be recognized, however, that these calculations are necessarily only approximations, since 1 or 2 moles of the reagent may combine with the primary amino groups of proteins. The former was assumed in the present calculations.

The nitrogen content of β-lactoglobulin[6] was depressed from 15.0 to 12.7 per cent through propylene oxide treatment. This corresponded to the introduction of 120 moles of propylene oxide into each mole of protein. However, the number of moles accounted for by protein group analyses was only 84.[7]

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[5] The apparent increases in the basic groups of proteins upon esterification are not yet understood.

[6] Determined after drying at 105° for 16 hours. When the moisture was determined separately and correction applied, the nitrogen content of the untreated egg albumin was 15.75 per cent. The apparent discrepancy appears to be due to the hygroscopicity of the material as recently emphasized by Chibnall, Rees, and Williams (17). Although the samples analyzed by the routine method were probably not dry, they have been regarded as containing similar moisture contents, since epoxide treatment does not affect the hydrophilic nature of proteins (see foot-note 1).

[7] These calculations are based on recent estimates of the molecular weights and of the number of carboxyl groups per mole for egg albumin and β-lactoglobulin, as summarized by Cohn and Edsall (3) (mol. wt. 45,000, with 51 carboxyl (including phosphoric acid) groups for egg albumin; mol. wt. 40,000, with 58 carboxyl groups for β-lactoglobulin). Data for the amino groups, tyrosine and cysteine, of egg albumin were taken from the same source. The corresponding values for β-lactoglobulin are those of Brand and Kassell (18).

The results of analyses for the chlorine content of epichlorohydrin-treated proteins which had not been electrodialyzed could be similarly interpreted. Electrodialyzed samples, however, contained considerably less chlorine than would be expected from their nitrogen content. It appeared probable that this procedure may have caused a loss through hydrolysis of part of the chlorine. The chlorine was also found to be removed readily by alkali.

Stability of Protein-Epoxide Bonds toward Acid and Alkali—It appeared of importance to ascertain the lability of the newly formed bonds toward acid or alkali, particularly in view of the fact that most protein group analyses were performed on samples that had been dissolved by means of acid or alkali or both. For this purpose various samples of propylene oxide-treated egg albumin were dissolved in 0.01 or 0.025 N hydrochloric acid or sodium hydroxide,[8] at protein concentrations of 0.75 and 1.875 per cent respectively. The solutions had pH values of 2.4 and 11.5. The numbers of free tyrosine, thiol, amino, and carboxyl groups were determined immediately and again after 1 or 2 days incubation at 40°.

Comparison of these values indicated in general little change.[9] A slight hydrolysis of esterified carboxylic esters seemed to occur in about half of the experiments, but the changes were of the same order as the error of the method used (10 per cent). An increase of the free phenolic groups was observed only in one out of seven experiments. The primary amino groups were found to be partly regenerated in alkali, but never in acid. This alkali lability of some isopropanol-amino linkages also became evident upon comparing the amino nitrogen content of acid-dissolved with that of the directly alkali-dissolved protein derivatives; the latter value was consistently higher than the former (about 15 *versus* 5 per cent of the amino nitrogen of untreated egg albumin). A similar, though inverse, relationship seemed to hold for a small fraction of the carboxyl groups; these appeared to be released immediately upon solution in acid (at pH 2.5 to 3.0). This could be demonstrated with protein samples that had been treated with propylene oxide in alkaline solution (Method 3) and could thus be dissolved directly in alkali. It was also indicated by comparing the free acid groups of a preparation obtained by Method 4, when dissolved in the minimum amount of acid (1.67 ml. of 0.1 N HCl per gm. of protein, pH 5.5) and when dissolved in the usual amount (13.3 ml. of HCl, pH 2.4). In these experiments about 10 per cent more free carboxyl groups was found in the samples exposed to pH 2.4 than in those dissolved at pH 5.5 or 11.

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[8] Samples not directly soluble in alkali were first dissolved in acid, and then treated with the necessary amount of alkali, added rapidly.

[9] The thio ether bonds of epoxide-treated proteins thus differ in their stability from the previously described thioacyl bonds (19).

SUMMARY

Epoxides, such as ethylene oxide, propylene oxide, and epichlorohydrin, were found to be suitable reagents for the esterification of protein carboxyl groups in aqueous solution at room temperature.

Through treatment of crystalline egg albumin and β-lactoglobulin with these compounds, preparations of modified protein were obtained which differed from the original material in that:

1. The derived proteins showed isoelectric points which had been shifted as much as 3 pH units toward the alkaline side.
2. They were insoluble in the isoelectric region, both in distilled water and in salt solutions.
3. They were more soluble on the acid side than on the alkaline side of the isoelectric point.
4. They contained considerably fewer free carboxyl, phenolic, primary amino, and sulfhydryl groups than the untreated proteins. The decreases of these various types of groups varied greatly, but in a predictable manner, depending upon the conditions of treatment.
5. Their nitrogen contents depended upon the type and length of treatment. The lowest values observed with the two proteins were 13.4 and 12.7 per cent of nitrogen, which indicated the introduction of approximately 80 and 120 moles of reagent per mole of egg albumin and β-lactoglobulin, respectively. In the case of egg albumin, the calculated figure agreed with that to be anticipated from the number of substituted groups as found by the various analyses. With β-lactoglobulin the number of reagent residues accounted for by group analyses was lower than that indicated by nitrogen analysis.

The only property of the proteins which was not appreciably affected by the treatment was the number of their total basic groups. Thus any difference in the basic character of the original amino groups and of the newly formed imines did not reveal itself under the conditions of the test.

In general, the newly introduced bonds were surprisingly stable in acid and alkaline solution with the exception of small proportions of the substituted carboxyl and amino groups which were hydrolyzed readily in acid and alkali, respectively.

The author is greatly indebted to H. S. Olcott of this Laboratory for valuable suggestions and criticism. The technical assistance of Mitzi Cooper is gratefully acknowledged.

Addendum—Treatment of a solution of egg albumin with propylene oxide at 95° (in a sealed tube) for 8 hours yielded one-half of the protein as a water-soluble derivative containing twice as many isopropanol residues as could maximally be introduced at room temperature. A considerable decrease in the total number of basic groups was observed, besides the usual loss of carboxyl and phenolic groups, which suggested the possibility that these groups might have become completely substituted under such conditions. The effects of epoxide treatment at elevated temperature will be further investigated.

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THE USE OF DYES FOR THE DETERMINATION OF ACID AND BASIC GROUPS IN PROTEINS

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(Received for publication, March 4, 1944)

Only a few methods are available for the determination of acid and basic groups of proteins. Titration curves have been most generally used for this purpose. Their application is restricted, however, to proteins which are either soluble over a wide pH range (1, 2) or completely insoluble (3, 4); in addition, considerable amounts of material are needed. The usefulness of titration curves is also limited by difficulties of interpretation. Metaphosphoric acid has recently been suggested as a reagent for the determination of basic protein groups with which it combines stoichiometrically in acid solution (5).

Acid and basic dyes are known to combine with protein groups of opposite ionic charge. The pioneer work of Loeb (6) was carried further by Chapman, Greenberg, and Schmidt (7–9) who measured the amounts of acid and basic dyes bound by proteins at various pH values and correlated their findings with those of titration studies. The present paper reports analytical methods for the determination of the total acid and basic groups of proteins based upon this ability to combine with dyes in buffered alkaline or acid solutions. The technique is rapid and simple and is applicable to both soluble and insoluble proteins; it is based on the photoelectric determination of the uncombined dye and therefore can be carried out with protein samples as small as the accuracy of weighing permits.

EXPERIMENTAL

Reagents—

Dye solutions; 0.1 per cent orange G (Coleman and Bell) and 0.2 per cent safranine O (National Aniline) in water.

The orange G, while labeled as of 78 per cent dye content, was found to be of constant chromogenic value and nitrogen and sulfur content after fractionation and recrystallization. Air-dried samples of both dyes contained approximately 10 per cent of water. Standard solutions were prepared from material dried to constant weight at 70° regardless of the "dye content" indicated on the labels.

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* This is one of four regional research laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

Buffers; pH 2.2, 980 ml. of 0.1 M citric acid and 20 ml. of 0.2 M disodium phosphate (10). pH 11.5, 250 ml. of 0.2 M disodium phosphate and 200 ml. of 0.1 N sodium hydroxide, water to 1000 ml. (11).

Determination of Orange G Bound by Proteins (Basic Groups)—To each of four 15 ml. test-tubes, suitable for use in an angle head centrifuge, are added 5 mg. of the protein, 1 ml. of pH 2.2 buffer, increasing amounts (1, 2, 3, or 4 ml.) of 0.1 per cent orange G, and two glass beads. If the protein dissolves in the buffer, it will be reprecipitated by the dye. The suspensions are shaken mechanically for 20 to 24 hours. They are then centrifuged, and aliquots of the supernatant solutions are diluted 100-fold. The color intensities are determined by means of a photoelectric colorimeter (Klett-Summerson), with a blue filter (Corning No. 038 + Pyrex No. 554). The dye concentration is read from a standard curve prepared from data obtained with appropriate dilutions of the orange G stock solution. This curve is a straight line in the range of 0.5 to 10 mg. of dye per liter. The readings are not affected by the presence of the acid buffer.

The dye bound by the protein is determined by subtracting the excess found in the supernatant from the amount added. Saturation of the protein with dye is indicated when no more dye is bound by the samples to which greater amounts of dye are added. For routine analyses a series of three samples is regarded as sufficient if the results agree within 10 per cent. If greater exactness is desired (as in all determinations listed in Tables I and II), six to twelve samples are prepared. More dye is used if a definite trend in the first series indicates that saturation has not been reached. The average of all values for the maximal amount of dye bound, in mg., multiplied by a conversion factor of 8.85[1] yields the results in terms of acid equivalents of dye bound per gm. of protein × 10^4.[2]

An alternate technique applicable to proteins soluble at pH 2.2 permits the analysis of only about 1.5 mg. of protein. For example, 1 ml. of a 0.75 per cent solution (in distilled water, dilute salt solution, or 0.01 N hydrochloric acid) is diluted with 1.5 ml. of the pH 2.2 buffer and four 0.5 ml. aliquots of this mixture are treated with 0.5 to 2.0 ml. portions of dye solution. The resulting precipitate is centrifuged off the following day and the supernatant treated as described above.

Determination of Safranine Bound by Proteins (Acid Groups)—The procedure is the same as that for the basic groups except for the use of the pH 11.5 buffer and the 0.2 per cent safranine solution. After 24 hours of shaking and subsequent centrifugation, the solutions are diluted 100- or 200-fold and read with the same blue filter used for orange G. The standard curve is a straight line for 0 to 10 mg. of dye per liter. The factor for calculating the results in terms of moles (= base equivalents) of dye bound per gm. of protein × 10^4 is 5.62.[3] Saturation of the protein with dye is again indicated by the lack of a progressive trend in the amounts of dye bound by samples treated with increasing amounts of dye solution. The results of at least three samples, agreeing within 10 per cent, are averaged.[2]

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[1] The factor represents the valence of the dye (2), × 10, divided by the molecular weight of the dye (452) × the amount of protein used (0.005 gm.). The empirical formula of orange G is $C_{16}H_{10}N_2O(SO_3)_2^{=}Na_2^{++}$.

[2] Analyses on proteins containing few basic or acid groups are necessarily inexact (±10 per cent) since they are derived from the differences between large figures. With proteins of typical composition, the averages of three or four samples can generally be reproduced within 5 per cent.

Of proteins soluble at pH 11.5, samples of only 1.5 mg. are needed, with a technique corresponding to that described above.

Effect of Variations in Experimental Conditions on Amounts of Dye Bound by Proteins. pH—For purposes of convenience and practicability, the protein-dye combination was carried out in buffered solutions, thus circumventing the need for pH measurements and adjustments. Buffers of pH 2.2 and 11.5 were selected because the work of Chapman, Greenberg, and Schmidt (7–9) had indicated that complete dissociation of protein groups in the presence of dyes was approached at these points. It did not seem advisable to use more strongly acid or alkaline buffers, since the advantages of more complete dissociation of protein groups might be overshadowed by the disadvantages of protein breakdown. Thus proteins treated with safranine at pH 12.0 bound considerably larger amounts of dye which increased with the excess added. This phenomenon which may be due to protein breakdown or to physical adsorption of the dye under these more extreme conditions was not further investigated.[4]

Concentration—When proteins were treated with an excess of orange G, the amount bound was independent of protein or dye concentration within the limits used (0.06 to 0.2 per cent protein and 0.05 to 0.1 per cent dye). Of safranine, slightly smaller amounts were bound at lower than at higher concentrations; thus the values obtained with solutions of 0.06 to 0.12 per cent protein concentration were 5 to 10 per cent lower than those obtained with 0.1 to 0.2 per cent protein concentration (dye concentration 0.12 to 0.16 per cent in both series).

When less than equivalent amounts of the dyes were added to dissolved proteins, the resulting protein-dye complexes were not completely precipitated. This resulted, paradoxically, in higher colorimetric readings in the soluble phase than when sufficient dye for saturation of the protein was added. This finding confirmed similar observations of Rawlins and Schmidt (9).

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[3] Safranine O is a mixture of homologous monoacid bases; the molecular weights of the two main constituents, $(C_{20}H_{19}N_4)^+Cl^-$ and $(C_{21}H_{21}N_4)^+Cl^-$, are 350.5 and 364.5. Calculations were based on a value of 355.

[4] Studies are in progress aiming at differential determination of the most strongly basic and acid groups by equilibrating proteins with the dyes in buffers less acid or basic than pH 2.2 or 11.5. A method for the determination of the approximate isoelectric point of insoluble proteins through measurement of the dyes bound at various pH levels will be described elsewhere.

Time, Shaking, State of Protein—For routine analyses, a 20 hour period was found practical. That equilibration was completed during that time was indicated by the fact that no greater amounts of dye were bound by proteins after 48 hours of interaction.[5] A shorter period may be sufficient for many purposes, since egg albumin was found to bind within 10 minutes 90 and 86 per cent of the maximal amounts of orange G and safranine, respectively.

Efficient shaking was essential for equilibration when protein samples were treated in test-tubes with more than 2 ml. of dye solution.

The state of dispersion of the protein did not greatly affect its capacity for the dyes. Thus insoluble proteins (keratins) of 60 to 80 mesh particle size bound only 10 to 20 per cent less dye than material which passed a 200 mesh screen. Also proteins which were insoluble in neutral solution but soluble in the buffers used bound the same amount of dye when dissolved in the buffer before addition of the dye as when solution was prevented by adding the dye before the buffer.

To investigate any possible effect due to incipient denaturation by the acid or alkaline buffer, samples of egg albumin were denatured by heating for 5 minutes to 70° at pH 2.5 or 11.7, and were subsequently analyzed for their dye-binding capacity. This was found to be unaffected by such treatment. That the heat treatment, but not the pH alone, led to appreciable denaturation was evident upon neutralization of aliquot samples.

Significance of Dye-Binding Capacity of Proteins—The amounts of the two dyes bound by proteins, expressed in terms of acid and base equivalents, have been regarded as indicative of the number of basic and acid protein groups dissociated under the conditions of the test at pH 2.2 and 11.5. To ascertain which types of groups were thus determined, several proteins were analyzed. Two of these, egg albumin and β-lactoglobulin, can be regarded as approximately pure proteins, inasmuch as the preparations had been repeatedly recrystallized and had been electrodialyzed. Lysozyme and insulin were crystalline preparations not electrodialyzed.[6] The others were commercial protein samples.

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[5] Wool, in contrast to other proteins studied including keratins and silk fibroin, did not reach an equilibrium with the dye solution within 20 to 24 hours.

[6] The preparations of crystalline egg albumin, β-lactoglobulin, and lysozyme were kindly placed at our disposal by Dr. F. E. Lindquist, Dr. E. F. Jansen, and Dr. H. L. Fevold, respectively; crystalline zinc insulin was supplied by Eli Lilly and Company.

A comparison of the number of basic groups which bind orange G with that number as determined by the methods of the literature (1, 2, 5, 12, 13) is summarized in Table I. The good agreement between corresponding values for the well characterized proteins may be regarded as evidence for the reliability of the proposed method. The approximate proportionality of results obtained with the crude proteins contributes additional support. It is concluded from these data that the number of basic groups binding orange G at pH 2.2 represents the sum of the guanidyl, imidazole, and amino (α- and ϵ-) groups of proteins.

TABLE I

Comparison of Basic Groups of Proteins As Determined by Various Methods

Protein*	Basic residues per gm. protein × 10^4			
	Dye-binding capacity†	Titration (1, 2, 12, 13)	Metaphosphoric acid-binding capacity (5)	Analysis or isolation (12, 13)
Egg albumin	8.8‡	8.0- 8.7	7.8- 8.0	9.0
β-Lactoglobulin	11.6‡	11.5		11.5
Casein	6.8	7.6- 9.0		8.0-9.4§
Fibrin	12.0			13.1§
Gelatin	6.0	8.9- 9.6		10.7§
Gliadin	4.3‡	3.4		4.4§
Insulin	9.4‡	10.1	10.3-11.0	9.5
Lysozyme	11.5‡	11.7-12.8		12.2
Zein	1.9	1.8- 2.1		1.5

* Egg albumin and β-lactoglobulin were electrodialyzed; all proteins were corrected for moisture content. Casein, gelatin, gliadin, and zein were commercial preparations. Two casein preparations gave identical values. See foot-note 6 concerning the other proteins.

† Moles of orange G bound at pH 2.2, ×2.

‡ These analyses represent averages of data obtained with 5 and 1.5 mg. protein samples, with protein concentrations ranging from 0.1 to 0.2 and from 0.06 to 0.15 per cent, respectively. Results of the two techniques agreed within 5 per cent.

§ Determined by nitrogen distribution.

A similar comparison of the groups binding safranine at pH 11.5 with the carboxyl and with the total acid (*i.e.* carboxyl + phenol + thiol) groups of several proteins is listed in Table II. The literature values given for most proteins must be regarded as rough approximations, inasmuch as they were calculated from incompletely confirmed determinations, by isolation, titration, and colorimetry, of the content of dicarboxylic amino acids, tyrosine, cysteine, and amide nitrogen. Only the data for egg albumin and β-lactoglobulin appear reliable, since they were obtained by recent improvements in analytical methods (14), supported by titration data

(1, 2). These two proteins showed a capacity to bind safranine in an amount which corresponded to their total acid groups, a finding which was supported by the data on most of the other proteins studied. It thus appears that the number of acid groups binding safranine O at pH 11.5 comprises the sum of the carboxyl, phenol, and thiol groups of proteins.[7]

Uses and Applications—The described micromethods for the routine determination of acid and basic protein groups were developed primarily for use in studies of protein derivatives. A considerable number of derivatives (of keratins, gluten, egg albumin, β-lactoglobulin, casein, etc.) has recently been prepared in this Laboratory by treatment with epoxides, aromatic isocyanates, anhydrides, aldehydes, nitrous acid, and combinations of these reagents. Epoxides were found to combine with both the acid and the primary amino groups of proteins (17). The esterification was demonstrated by the decreases in the number of acid groups of the

TABLE II

Comparison of Total Acid and Carboxyl Groups of Proteins with Their Capacity to Bind Safranine

Protein[*]	Dye bound[†]	Total acid groups[‡]	Carboxyl groups[‡]
Egg albumin	13.5[§]	13.8	10.4
β-Lactoglobulin	17.6[§]	17.5	14.5
Casein	19.4	16	13
Gelatin	12.7	10	10
Gliadin	5.5	7	5
Insulin	17.5	13	6
Zein	5.5	6	3

[*] See the corresponding foot-note to Table I.

[†] Moles of safranine O bound at pH 11.5 by 10^4 gm. of protein (range of protein concentrations, 0.1 to 0.2 per cent).

[‡] Of 10^4 gm. of protein; calculated from analyses for glutamic and aspartic acids, amide N, tyrosine, and cysteine as summarized by Cohn and Edsall (12), Chibnall *et al.* (14), and Brand and Kassell (15), and as amended for glutamic acid of insulin, gelatin, and gliadin by Olcott (16). The unknown number of terminal carboxyl groups of the polypeptide chains was disregarded.

[§] In more dilute solution (protein concentrations 0.06 to 0.15 per cent), 12.4 and 16.5 moles of dye were bound by egg albumin and β-lactoglobulin, respectively.

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[7] Before suitable conditions for the determination of total acid groups had been recognized, a relative measure of the acidity of various proteins and derivatives was obtained from their tendency to bind dyes when the amounts added were less than those needed for saturation. For these studies both safranine and methylene blue were used. The amounts of these dyes most readily bound yielded comparative data on the acidity of proteins and derivatives which have since been confirmed by determinations of their total acid groups.

derivatives, as estimated by dye methods, particularly with those of submaximal combination which are believed to measure primarily the carboxyl groups.[7] On the other hand, the introduction of alkoxy residues in the amino groups did not remove their basic nature. Actually a slight increase in the basic groups was found in many epoxide-treated proteins, a phenomenon which is not yet understood.

Intensive treatment of certain proteins with phenyl isocyanate led to introduction of the reagent to the extent of up to 30 per cent (by weight) of the protein.[8] Dye methods have now yielded information which may contribute to an accounting for this extent of interaction. A few typical results obtained with cattle hoof powder and rennet casein are listed in Table III. Treatment with phenyl isocyanate was found to cause a loss of almost all basic groups and of a considerable proportion of the acid groups of the proteins. On the other hand, phthalic anhydride was found to react only with part of the basic groups. In contrast to phenyl isocyanate, phthalic anhydride increased the number of acid groups, as would be expected from introduction of phthalic acid residues. With both reagents the observed decrease in the basic groups corresponded to the loss in amino nitrogen.[8] The increase in the acid groups of the deaminated protein may have been due to nitration of the phenol residues. Treatment with formaldehyde caused only minor changes in both acid and basic groups. This is

TABLE III

Effects of Various Reagents on Basic and Acid Groups of Proteins

Protein and treatment*	Basic residues†	Total acid residues†
Hoof powder, more than 200 mesh, untreated	8.8	10.4
" " 40–60 mesh, untreated	8.0	8.0
Phenyl isocyanate‡	0.0	2.1
Phthalic anhydride	3.6	13.3
Propylene oxide	9.2	8.7
Same, followed by phenyl isocyanate	0.0	1.7
Formaldehyde	7.1	11.2
Nitrous acid	3.8	13.0
Casein, untreated	6.8	20.3
Phenyl isocyanate	0.0	4.0
Propylene oxide	6.4	7.2

* See foot-note 8 for the methods of preparation and treatment.

† Per gm. of protein × 10^4. Determinations based on the capacity to bind orange G and safranine O at pH 2.2 and 11.5, respectively.

‡ All hoof derivatives were powders which passed a 200 mesh screen.

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[8] Fraenkel-Conrat, H., and Olcott, H. S., in preparation for press.

in contrast to a marked decrease in the primary amino nitrogen, indicating that the N-methylol or N-methylene groups resulting from the interaction of amino groups with aldehydes retain sufficient basicity to bind orange G at pH 2.2.

SUMMARY

Microanalytical methods were developed for the estimation of the number of acid and basic groups of proteins. These were based on the tendency of the polar groups to bind dyes of the opposite charge, resulting in a precipitation of the protein-dye complex. The acid dye, orange G, combined stoichiometrically with basic protein groups in a buffer of pH 2.2. The basic dye, safranine O, reacted with acid groups at pH 11.5, but the extent of combination was in this case slightly affected by other factors, such as protein concentration.

The number of protein groups binding these dyes corresponded well to the total number of basic (guanidyl, imidazole, amino) and acid (carboxyl, phenol, thiol) groups of crystalline egg albumin and β-lactoglobulin and, approximately, to those of several crude proteins studied.

The proposed micromethods were applicable to both soluble and insoluble proteins. They have proved useful tools in the interpretation of the action of various chemical agents on proteins.

The valuable suggestions and criticisms of H. S. Olcott of this Laboratory are gratefully acknowledged.

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A SYNTHESIS OF METHIONINE CONTAINING ISOTOPIC CARBON AND SULFUR*

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(Received for publication, March 30, 1944)

Methionine containing excess quantities of the stable isotopes S^{34} and C^{13} (in the β and γ positions) was desired for certain tracer experiments on the relation of methionine to cystine. The isotopic carbon and sulfur were initially in the form of NaCN and Na_2SO_3 (1, 2) containing various amounts of C^{13} and S^{34} respectively.[1] The compounds and reactions involved in the synthesis are shown in Diagram 1.

The preparation of methionine from benzyl mercaptan and ethylene chloride or bromide had been worked out by Patterson and du Vigneaud (3, 4) and modified for use with S^{35} by Tarver and Schmidt (5). With the exception of the first step, which involves condensation of sodium benzyl mercaptide with *5 to 10 moles* of ethylene chloride or bromide to form benzyl β-chloroethyl sulfide (III), these procedures give good yields, and are well suited to use with valuable isotopic materials. On the comparatively small scale of operation (10 gm.) used in the present work, direct recovery of isotopic ethylene halide, to which resort was made in the synthesis of methionine-β,γ-d_2 (4), did not seem practical. Consequently, a device (illustrated in Fig. 1) for continuous circulation of ethylene chloride was used. Unchanged ethylene chloride was constantly kept in excess in the reaction cup (*G*) to which isotopic benzyl mercaptide in propyl alcohol was slowly added. By use of equivalent quantities of benzyl mercaptan-S^{34} and ethylene chloride-C^{13} (II) a yield of 58 per cent of reasonably pure benzyl β-chloroethyl sulfide (III) was obtained.

Ethylene chloride was prepared from the heavy sodium cyanide, by modification of the standard reactions outlined in Diagram 1, in over-all yield of about 46 per cent. Methylation of cyanide with dimethyl sulfate (6) instead of methyl iodide was found unsatisfactory. Ethylene, generated by pyrolysis of the quaternary ammonium base (I), was collected at low temperature in liquid chlorine diluted with methylene chloride.

The preparation of benzyl mercaptan-S^{34} is also illustrated in Diagram 1. After oxidation of the sulfite to sulfate, use was made of the St. Lorant reagent (5, 7) to produce H_2S^{34}, which was collected in acid iodine solution as free S^{34} in almost quantitative yield. Treatment of excess benzylmagnesium chloride with this sulfur gave isotopic benzyl mercaptan in approximately 88 per cent yield.

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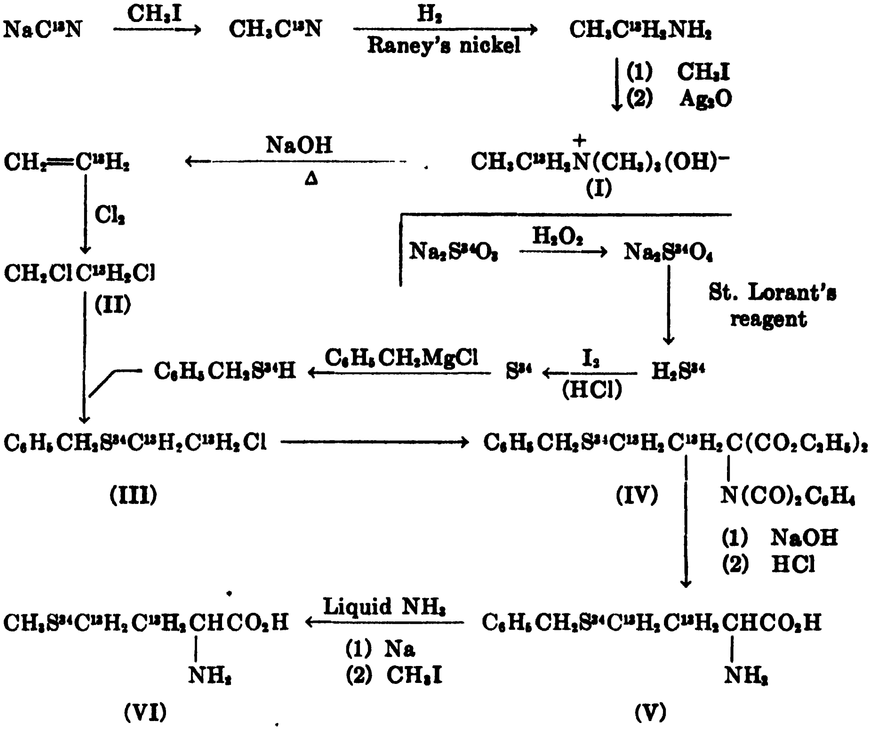
* The experimental work in this paper was carried out during the period of 1940–41. The presentation of the work has been unavoidably delayed.

[1] We wish to express our appreciation to Professor H. C. Urey of Columbia University from whom we obtained the isotopic carbon and sulfur samples.

Tarver and Schmidt (5) have prepared radioactive benzyl mercaptan in 70 per cent yield by the action of benzyl chloride on potassium bisulfide. Since our work was completed, Seligman, Rutenburg, and Banks (8) have



$NaC^{13}N \xrightarrow{CH_3I} CH_3C^{13}N \xrightarrow[\text{Raney's nickel}]{H_2} CH_3C^{13}H_2NH_2$
(1) CH_3I
(2) Ag_2O
$CH_3C^{13}H_2\overset{+}{N}(CH_3)_3(OH)^-$
(I)
NaOH
Δ
$CH_2{=}C^{13}H_2$
Cl_2
$CH_2ClC^{13}H_2Cl$
(II)
$Na_2S^{34}O_3 \xrightarrow{H_2O_2} Na_2S^{34}O_4$
St. Lorant's reagent
H_2S^{34}
I_2
(HCl)
S^{34}
$C_6H_5CH_2MgCl$
$C_6H_5CH_2S^{34}H$
$C_6H_5CH_2S^{34}C^{13}H_2C^{13}H_2Cl$
(III)
$C_6H_5CH_2S^{34}C^{13}H_2C^{13}H_2C(CO_2C_2H_5)_2$
$N(CO)_2C_6H_4$
(IV)
(1) NaOH
(2) HCl
$C_6H_5CH_2S^{34}C^{13}H_2C^{13}H_2CHCO_2H$
NH_2
(V)
Liquid NH_3
(1) Na
(2) CH_3I
$CH_3S^{34}C^{13}H_2C^{13}H_2CHCO_2H$
NH_2
(VI)

Diagram 1. Compounds and reactions involved in synthesis of methionine. Compound III was of course in reality a mixture of equal quantities of $C_6H_5CH_2S^{34}$-$CH_2C^{13}H_2Cl$ and $C_6H_5CH_2S^{34}C^{13}H_2CH_2Cl$. The same holds for Compounds IV, V, and VI.

reported the use of radioactive elemental sulfur and benzylmagnesium chloride with a result similar to ours.

EXPERIMENTAL

Isotopic methionine, $CH_3S^{34}C^{13}H_2C^{13}H_2CHNH_2COOH$, was prepared from $NaC^{13}N$ and $Na_2S^{34}O_3$ of various isotope concentrations by a combination of the following procedures.

Sulfur—15.0 gm. of Na_2SO_4 were placed in a 1 liter Claisen flask suitably altered to accommodate the absorption flasks which will be described subsequently. A solution of 16.5 cc. of concentrated ammonia in 200 cc. of water was added to the flask. To the cooled solution were added, during 5 minutes, 25 cc. of 30 per cent hydrogen peroxide. After 20 minutes the solution was evaporated *in vacuo* on a water bath.

The solid was rinsed down from the walls of the flask with a few cc. of water and, after attachment of the absorption train, 25.1 gm. of red phosphorus and a mixture of 167 cc. of colorless 57 per cent HI and 125 cc. of anhydrous formic acid were added (7). The H_2S was absorbed in a trap cooled in ice and containing 234 cc. of 1 N iodine in potassium iodide and 19.5 cc. of concentrated HCl. This trap was followed by three auxiliary traps containing smaller amounts of the iodine-HCl solution.

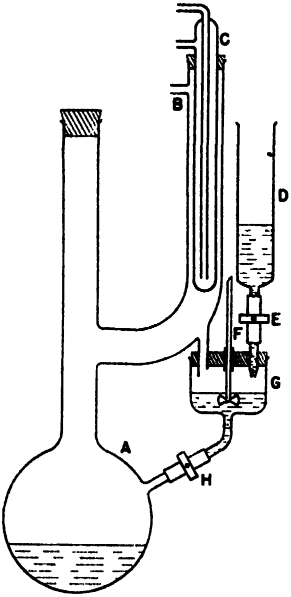


FIG. 1. Apparatus for condensation of $C_6H_5CH_2S^{34}H$ with $C^{13}H_2ClCH_2Cl$. *A* represents the bulb in which is placed the ethylene chloride; *B*, a vent; *C*, the condenser; *D*, the dropping funnel; *E*, a pinch-clamp; *F*, a mechanical stirrer; *G*, a cup that receives material from the condenser; *H*, a pinch-clamp.

The flask containing the sulfate and reducing mixture was heated in an oil bath to 90° with a slow stream of nitrogen passing through the apparatus; after 1 hour at this temperature the bath was heated during successive hours to 110–115° and to 140–150°, where the temperature was maintained for 1 hour. After this treatment a negative test for sulfide in the vapor over the reducing mixture was obtained.

The contents of the scrubbers were combined, cooled in ice, and stannous chloride (40 per cent in 1 N HCl) was added gradually with stirring until all the iodine color disappeared. The gummy sulfur was separated and washed as free as possible from liquors. After being dried overnight *in vacuo*, the brittle product was ground with dilute acidified stannous chloride solution, with dilute HCl, and finally with water. The dried powder (95.5 per cent S by analysis) weighed 3.91 gm.; this represents 99 per cent of the theoretical yield from Na_2SO_3.

Benzyl Mercaptan—3.8 gm. of sulfur (95.5 per cent) were added during 45 minutes to a well stirred benzylmagnesium chloride solution prepared from 8 gm. of magnesium, 34.3 cc. of benzyl chloride, and 160 cc. of ether. The reaction flask was cooled in ice and the orifice flushed with a constant stream of pure nitrogen. After addition of the sulfur, the pasty mixture was stirred 1 hour at room temperature, 100 cc. of dry benzene were added, and the stirring was continued for an hour longer at ordinary temperature and then for 2 hours at 50–60° under a reflux.

The material was cooled in an ice-salt bath and hydrolyzed by slow addition of 15 per cent HCl. After separation of the layers and extraction of the aqueous phase with benzene, the combined organic layers were distilled. Traces of sulfide evolved were absorbed in alkali. Distillation of the residue at 18 to 20 mm. yielded at 85–87° 12.5 gm., or 88 per cent of the theoretical yield, of benzyl mercaptan.

Ethylamine—11.3 gm. of sodium cyanide (96 per cent) were dissolved in 17 cc. of water, 36.3 gm. of methyl iodide were added, and the mixture was shaken for 24 hours (9). After overnight storage in a refrigerator, a little water was added to dissolve a small precipitate and the material was distilled very slowly. The fraction from 76–100° (13.8 gm.) was rinsed into a hydrogenation bottle containing 10 gm. of Raney's nickel paste, and was diluted to 75 cc. (10). Approximately the theoretical amount of hydrogen had been absorbed after 4 hours of shaking. The ice-cold solution was filtered and the nickel washed very thoroughly with water and 5 N NaOH (total of ten washings). Preparation of N-ethyl-*p*-bromobenzenesulfonamide on a small aliquot indicated a yield of approximately 70 per cent from NaCN.

Ethylene Chloride—11 gm. of ethylamine in about 10 per cent aqueous solution were placed in a 1 liter 3-necked flask bearing a stirrer, efficient condenser, and dropping funnel. The solution was cooled in ice and 23.6 gm. of NaOH were added in portions (11). After removal of the ice bath 65 cc. of methyl iodide were added in portions of 3 to 5 cc., at approximately the rate at which the iodide was consumed. An ice bath was applied occasionally to prevent more than a gentle reflux. Methyl iodide collected in the reaction flask toward the end and a little more NaOH had to be added to keep the mixture alkaline. The material was stirred for 1 hour at room temperature and then refluxed and stirred for 2 hours. At the end of this time, the excess methyl iodide was distilled. The Ag_2O freshly prepared from 220 gm. of $AgNO_3$ was slowly added to the solution which was shaken and cooled. After filtration, the AgI was washed four times by suspension in distilled water. The combined filtrates were concentrated at 70° and 15 mm. to 200 cc.

The concentrated solution was filtered into a 250 cc. distilling flask sealed to a down distillation condenser and two receivers in series, cooled in ice, and so arranged that the evolved gas bubbled through an excess of 6 N H_2SO_4. To the second receiver were sealed in series four bubble traps which were cooled in a dry ice-trichloroethylene bath. The first served to condense moisture and the other three each contained 7 cc. of methylene chloride and 7 cc. of dry chlorine for absorption of the ethylene (12). The vent on the last trap was connected to a $CaCl_2$ tube and a bubble counter. The system was all-glass and arranged to permit nitrogen to sweep through, the slightly elevated internal pressure being indicated by a mercury U-tube. At a bath temperature of 130–140°, the water was distilled from the solution in the distilling flask into the acid in the receivers. After 4 hours at 140–150°, at which temperature most of the ethylene was evolved, the bath was raised gradually to 210° and finally to 250° for a few minutes. Back titration of the sulfuric acid indicated that nearly the theoretical amount of the base had been evolved. The chlorine traps were unsealed and the chlorine was allowed to evaporate at room temperature. The residual materials were combined, cooled in ice, and one-half saturated $NaHSO_3$ solution was added with shaking until the chlorine color disappeared. After the solution was dried over $MgSO_4$ and filtered, the methylene chloride was distilled very slowly. The intermediate fractions were redistilled. 19.3 gm. of material boiling at 77–86° were obtained; this crude material represents 80 per cent of the theoretical yield from the ethylamine.

Benzyl β-Chloroethyl Sulfide—Fig. 1 represents the apparatus used to condense sodium benzyl mercaptide with molar proportions of ethylene chloride. In the 125 cc. bulb (*A*) were placed 8.9 gm. of ethylene chloride. The dropping funnel (*D*) which was controlled by the rubber connection and pinch-clamp *E* contained 2.2 gm. of sodium and 11.8 gm. of benzyl mercaptan in 50 cc. of dry *n*-propyl alcohol; the materials were protected from atmospheric moisture by $CaCl_2$ tubes on the mouth of *D* and on the vent (*B*). At a bath temperature of 100–150° the accumulated *n*-propyl alcohol and unchanged ethylene chloride in *A* refluxed from the condenser (*C*) and dropped into the 15 cc. cup (*G*); the product, being relatively non-volatile, remained in *A*. The level of NaCl suspension in *G* was controlled by the rubber connection and pinch-clamp *H*. The contents of the cup were mixed mechanically with the stirrer (*F*); about 10 hours were required for the addition of the mercaptide solution.

When all the mercaptide had been added, the material was poured into 400 cc. of water and after separation the aqueous phase was extracted four times with 25 to 50 cc. portions of benzene. The organic layers were combined and the solvent was distilled finally *in vacuo*. The residue was distilled at 1.5 mm., yielding 9.8 gm., *i.e.* 58 per cent of the theoretical yield, of benzyl β-chloroethyl sulfide distilling at 105–108° (3).

Another experiment indicated that 1-chloro-2-bromoethane could be used equally well for the preparation of benzyl β-chloroethyl sulfide, but that it offered no advantage as far as yield is concerned over the more easily prepared ethylene dichloride. 10 gm. of benzyl mercaptan were added to a solution of 1.9 gm. of sodium in 45 cc. of absolute alcohol. This cooled solution was poured into 11.5 gm. of ice-cold 1-chloro-2-bromoethane; after a few seconds there was a sudden precipitation in the solution. After agitation for 5 minutes at room temperature, the mixture was poured into 350 cc. of water, the layers were separated, and the aqueous phase extracted with benzene. After distillation of the benzene and a small forerun, 8.5 gm. of the product, representing 56 per cent of the theoretical yield, were obtained at 107–109° and a pressure of 1.5 mm. A qualitative test for bromine on this product was negative, and the boiling point agrees fairly well with that reported for the chloro compound.

S-Benzylhomocysteine—This compound was prepared by a modification of the method of Tarver and Schmidt (5). 8.3 gm. of benzyl β-chloroethyl sulfide, 15.7 gm. of ethyl sodiophthalimidomalonate, and 1 to 2 cc. of dry toluene were placed in a 100 cc. centrifuge tube which was closed with a $CaCl_2$ tube. The mixture was heated 5 hours at 170° in an oil bath, was centrifuged and decanted, and the NaCl was washed repeatedly with toluene. After evaporation of the solvent *in vacuo*, the oil was heated for 2 hours in a boiling water bath with 19.5 cc. of alcohol and 50.5 cc. of 5 N NaOH. The lumps of solid which formed were crushed as well as possible at intervals during the heating. The mixture was cooled in ice and the solid was collected and washed twice with alcohol-water (15:1 cc.). The filtrate was evaporated *in vacuo* to a thick paste and the solid collected and washed as above. The total crude sodium salt (21.4 gm.) thus obtained, 222 cc. of water, and 26.3 cc. of concentrated HCl were heated on a steam bath for 2 hours, after which time 135 cc. more of concentrated HCl were added, and the heating was continued for 45 minutes. The cooled mixture was treated with 1 gm. of infusorial earth and 1 gm. of decolorizing carbon to remove oil, and was filtered. After concentration to about 125 cc., the filtered solution was cooled in ice and made alkaline to Congo red and acid to litmus with ammonia. The white precipitate was collected after a few hours in the refrigerator and was washed with ice water, followed by alcohol and ether. The yield was 7.0 gm., representing 70 per cent of the theoretical yield from benzyl β-chloroethyl sulfide.

Methionine—Benzylhomocysteine was converted to methionine by the method of Patterson and du Vigneaud (4). The over-all yields of analytically pure methionine from sodium cyanide and sodium sulfite were about 5.5 and 11 per cent of the theoretical yields, respectively. A sample obtained by mixing the products from two separate complete syntheses contained[2] 1.67 atom per cent excess C^{13} and 4.2 atom per cent excess S^{34}. Concentrations of these orders of magnitude were anticipated from the isotope content of the starting materials.

SUMMARY

dl-Methionine containing an excess of the stable isotopes C^{13} (in the β and γ positions) and S^{34} has been prepared from isotopic sodium cyanide and sodium sulfite.

The authors wish to express their appreciation to Dr. J. R. Rachele of this laboratory for carrying out certain microanalytical work in connection with this problem.

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[2] The isotopic content of the synthetic methionine was determined by Dr. D. Rittenberg of the College of Physicians and Surgeons, Columbia University, to whom we are greatly indebted.

COMPONENT FATTY ACIDS OF EARLY AND MATURE HUMAN MILK FAT*

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(Received for publication, April 10, 1944)

The techniques of qualitative and quantitative fatty acid analysis have been applied frequently to the fat of cow's milk and the milk of a few other animals. However, there is no comparable information available on human milk.

The most extensive attempt to characterize human milk fat was that of Bosworth (1). His data, based on an analysis of 3 pounds of fat, indicated that there are several differences of both a qualitative and quantitative nature from the fat of cow's milk. Thus, he comments that "the fatty acids of low molecular weights are present in the fat from human milk in much smaller quantities than in the fat from cow's milk." Unfortunately, it is difficult to calculate from Bosworth's data the composition of the thirty fractions he obtained in the distillation of the mixed methyl esters.

The present study on breast milk was undertaken as part of a general program sponsored by the Nutrition Foundation, Inc., to provide more satisfactory quantitative information on the composition of human milk.

Samples of human milk for this study were collected during several periods of lactation and dried by the cryochem process at the Children's Fund of Michigan. Two of the samples studied were taken from 75 individuals during the first 3 days post partum and the third sample represents normal milk taken from two individuals during the 22nd to 43rd day of lactation.

EXPERIMENTAL

Preparation of Fat—The lipid material was extracted from the cryochem-dried samples of colostrum and mature milk first with ethanol and then with ethyl ether in a Soxhlet apparatus for a total of 96 hours. After removal of the solvents, the combined extracts from each extraction were taken up in redistilled ethyl ether and washed free of water-soluble impurities. The water washings and extracted residues were saved for further studies. Aliquots of the dried ether solution of the total lipid were weighed after removal of solvent *in vacuo* to determine the amounts of fat extracted from each dried milk sample. The completeness of the original extractions was checked by petroleum ether extraction of a saponified and acidified portion of the extracted residues from each sample. The amount of residual lipid present was, in each case, less than 0.4 per cent of the original amount present. A summary of the fat content of the milks is given in Table I.

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† Nutrition Foundation Fellow.

Phospholipid Separation—The ether solutions of the total lipids were concentrated to approximately 75 ml. and poured with stirring into 1200 ml. of dry redistilled acetone at room temperature. 1 ml. of magnesium chloride solution (saturated solution in alcohol) was added The mixture was allowed to stand overnight and the flocculent precipitate which separated was dissolved in moist ether and reprecipitated with acetone. After filtration, the residue was again taken up in moist ether and the magnesium chloride removed by centrifugation. The ether solution was dried with anhydrous sodium sulfate, solvent was removed *in vacuo*, and the phospholipid was weighed. Phospholipid analyses are shown also in Table I.

TABLE I

Lipid Content of Human Milks

Analysis	Colostrum		Mature milk, 22nd to 43rd day
	1st and 2nd days	3rd day	
Sample extracted, *gm*.	121.2	183.4	181.2
Fat obtained, *gm*.	19.66	35.76	45.90
Total lipid on water-free basis, %	16.8	20.1	26.2
" " " whole milk " %	2.2	2.3	3.2
Phospholipid of total lipid, %	6.1	1.7	0.5
" " whole milk, %	0.80	0.19	0.06

Phosphorus determinations according to the method described by Fontaine (2) indicated an average phosphorus content in the phospholipids of 3.65 per cent.

Volatile Fatty Acids—The acetone-soluble lipids from each milk sample were saponified in redistilled methanol and the resulting soaps were reduced nearly to dryness *in vacuo* and transferred with distilled water to an all-glass steam distillation apparatus. After acidification of the soaps with 50 per cent sulfuric acid, the free acids were steam-distilled for 6 hours until at least 1200 ml. of distillate were collected. In order to avoid any loss of volatile acids, the condensate was collected in an ice-cooled receiver. The aqueous distillate was extracted with ethyl ether and dried over neutral anhydrous sodium sulfate. After removal of the ether, the steam-volatile acids were fractionally distilled through a fractionating column. The recovered ether, the extracted aqueous solution, and the sodium sulfate used for drying were all titrated with standard KOH, and the total acidity was calculated as butyric acid. Calculation of the compositions of the distillate fractions was made on the basis of the neutralization equivalents and iodine values of the individual fractions. The data for the distilled volatile fractions from the analysis of mature milk fat are given in Table II. The calculated composition of the volatile acids of the three samples studied was 2.8 moles per cent for human colostrum on the 1st and 2nd days; 2.0 on the 3rd day; 3.4 for mature human milk.

The percentage of volatile acids found, compared with the percentages in butter fat (3), suggested the possibility that, in spite of all the precautions taken, loss of low molecular weight acids might have occurred either during the cryochem drying or subsequent handling. In an effort to check the entire procedure used in these analyses of human milk fat, a sample of unpasteurized cow's milk (obtained on the market in Detroit) was dried by the cryochem process exactly as the human milk samples had been dried. The subsequent extraction and volatile acid analysis were performed in a manner identical to that described above for the human milk fats. The results of this analysis indicated the presence of an amount of steam-volatile acids (14.5 moles per cent) which would be expected on the basis of earlier analyses of cow's milk fat.

TABLE II

Fractional Distillation of Steam-Volatile Acids (Mature Milk)

Fraction No	Weight	Neutralization equivalent	Iodine value
	gm.		
1*	1.176		
2*	0.373		
3	0.090	139.5	0.0
4	0.097	186.0	7.6
Residue	0.299	195.3	8.1

* Ethyl ether; butyric acid calculated from titration of all recovered ether, 3 mg.; sodium sulfate used for drying, 16 mg.; ether-extracted aqueous solution, 105 mg.

Non-Volatile Fatty Acids—The acids non-volatile in steam were converted to neutral methyl esters, without the customary resort to separation by either crystallization of the acids or lead salts, and fractionally distilled at 1 to 2 mm. head pressure through a packed column (4) fitted with a take-off designed to separate adjacent fractions with minimum admixture (Fig. 1). Details of the analysis of the ester fractions from one of the distillations (mature human milk fat) are given in Table III. Analyses of the two other samples were very similar to the one shown. The percentages of octadecadienoic, octadecatrienoic, and eicosatetraenoic acids were obtained by spectrophotometric examination of an aqueous solution of the soaps after isomerization in ethylene glycol at 185° for 30 minutes. Calculations of the component acids were made from these data by the customary procedures (5) for the first twelve fractions. For Fractions 13 to 16 the increment of the iodine value due to the determined amounts of octadecadienoic and octadecatrienoic acid was calculated and subtracted from the determined iodine value to obtain the amount of oleic acid present. The equivalent weight of the saturated acids present was then calculated to establish the proportions of individual saturated acids. In the case of fractions containing C_{20} esters, the C_{18} material (saturated and unsaturated) was as-

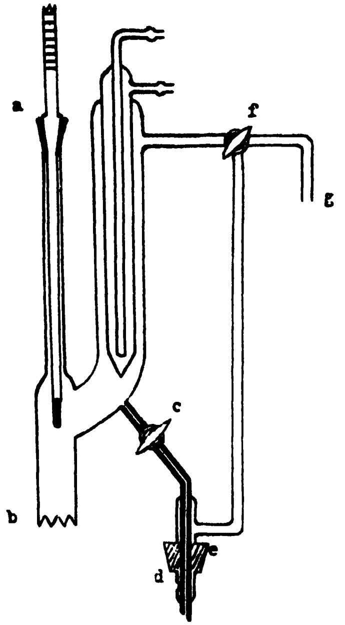

FIG. 1. Distillation head for fractional distillations under reduced pressure. This apparatus provides for the interchange of the receiving tubes without alteration of the pressure on the column itself and for minimum mixing of the distillate during collection. (*a*) Standard taper 10/30 250° thermometer with a 6 inch stem from the joint to the bulb. (*b*) The distillation head may be sealed directly to a packed fractionating column or there may be a standard taper connection to make the head interchangeable. (*c*) 3-Way capillary stop-cock with one outlet through the plug, sealed to within 1 cm. of the bowl at a point immediately under the tip of a sealed-in finger condenser; the total distance of the capillary should be kept at a minimum (*d*) Small hole for air inlet during exchange of receivers. (*e*) Either standard taper connection or rubber stopper to hold interchangeable receivers. (*f*) 3-Way T stopcock (2 mm. bore) connected to 10 mm. glass tubing. (*g*) Connection to vacuum line.

sumed to have the same composition as in the main C_{18} fraction (No. 15). The amount of eicosatetraenoic acid was calculated from spectrophotometric analysis[1] and the remainder of the C_{20} material present was calculated as eicosanoic acid and eicosadienoic acid. The basis for calculation of the latter acid was the large amount of absorption due to diene conjugation other than that arising from octadecadienoic and eicosatetraenoic acids which were found by spectral analysis of the isomerized soaps of fractions containing C_{20} material. The total component fatty acids of the mature human milk fat and the two samples of colostral fat are given in Table IV.

TABLE III

Fractional Distillation of Methyl Esters of Non-Volatile Fatty Acids from Mature Human Milk

Fraction No.	Amount	Saponification equivalent	Iodine value (Wijs)	Octadecadienoic acid	Octadecatrienoic acid	Eicosatetraenoic acid
	gm.			*weight per cent*	*weight per cent*	*weight per cent*
1	0.302	183.0	3.9			
2	0.574	204.5	2.1			
3	0.588	209.2	1.4			
4	0.497	214.8	1.8			
5	0.362	230.5	4.9			
6	0.801	236.9	6.0			
7	1.087	239.9	4.2			
8	1.023	246.0	8.1			
9	0.765	261.7	17.2			
10	3.036	268.7	13.3			
11	1.275	270.4	8.8			
12	2.310	270.2	9.0	0.2	0.0	
13	5.595	286.3	71.2	13.57	0.5	
14	1.932	294.7	94.6	17.44	1.0	
15	10.978	295.8	83.5	13.44	0.7	
16	1.057	302.5	87.6	13.74	0.7	6.2
17	0.920	316.0	130.2			15.3
18	0.315	348.4	143.0			17.6
19 (Residue)	0.558	364.1*	94.5			10.4

* After removal of unsaponifiable material.

Absorption curves for the isomerized soaps of four fractions from the distillation of the methyl esters of mature milk fat are shown in Fig. 2. On the basis of the determined equivalent weight, iodine value, and spectrophotometric study of the isomerized soaps, Fraction 14 contains only C_{18} material; Fraction 15 is mainly C_{18} with a small amount of C_{20}; and Fractions 17 and 18 are almost entirely C_{20}, or higher, esters. Tetraene material is evidenced in Fractions 15, 17, and 18 by the typical absorption maxima at four wave-lengths. The triene absorption at 270 mμ can be accounted for almost completely by the tetraenoic acids. However, the extensive absorption at 234 mμ, representing diene conjugation, cannot possibly have arisen entirely from the eicosatetraenoic acid present and can be due, in part, only to a diene C_{20}, a C_{22} acid, or both, since the amount of octadecadienoic acid present in the last fractions is very small and triene

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[1] Standard absorption values for eicosatetraenoic acid were graciously provided by Dr. B. W. Beadle and Dr. H. R. Kraybill, American Meat Institute, Chicago, Illinois.

TABLE IV

Component Fatty Acids of Human Milk Fats

Acids		Weight percentage			Molar percentage		
		1st and 2nd days	3rd day	Mature	1st and 2nd days	3rd day	Mature
Saturated	Butyric	0.2	0.3	0.4	0.7	0.8	1.1
	Caproic	0.1	0.1	0.1	0.3	0.2	0.1
	Caprylic	0.8	0.1	0.3	1.5	0.1	0.6
	Capric	3.5	0.9	2.2	5.3	1.4	3.3
	Lauric	0.9	2.6	5.5	1.2	3.4	7.1
	Myristic	2.8	4.9	8.5	3.3	5.7	9.6
	Palmitic	24.6	27.8	23.2	25.4	28.9	23.4
	Stearic	9.9	7.7	6.9	9.2	7.2	6.3
	As arachidic	4.9	2.7	1.1	4.1	2.3	0.9
Unsaturated	Decenoic	0.2	0.1	0.1	0.3	0.1	0.1
	Dodecenoic	0.1	0.1	0.1	0.1	0.1	0.1
	Tetradecenoic	0.1	0.2	0.6	0.1	0.2	0.7
	Hexadecenoic	1.8	2.9	3.0	1.9	3.0	3.0
	Octadecenoic	36.0	37.1	36.5	33.8	35.1	33.3
	Octadecadienoic	7.5	6.2	7.8	7.1	5.9	7.2
	Octadecatrienoic	0.3	0.3	0.4	0.3	0.2	0.4
	Eicosatetraenoic	1.8	1.6	0.9	1.5	1.4	0.8
	As eicosadienoic	4.6	4.7	2.4	3.9	4.0	2.0
Total	Saturated	47.7	47.0	48.2	51.0	50.0	52.4
	Unsaturated	52.3	53.0	51.8	49.0	50.0	47.6
	C_4	0.2	0.3	0.4	0.7	0.8	1.1
	C_6	0.1	0.1	0.1	0.3	0.2	0.1
	C_8	0.8	0.1	0.3	1.5	0.1	0.6
	C_{10}	3.7	0.9	2.3	5.6	1.5	3.4
	C_{12}	0.9	2.6	5.6	1.2	3.4	7.2
	C_{14}	2.9	5.1	9.1	3.4	5.9	10.3
	C_{16}	26.4	30.7	26.2	27.3	31.9	26.3
	C_{18}	53.7	51.3	51.6	50.4	48.4	47.3
	As C_{20}	11.3	9.0	4.4	9.6	7.8	3.7

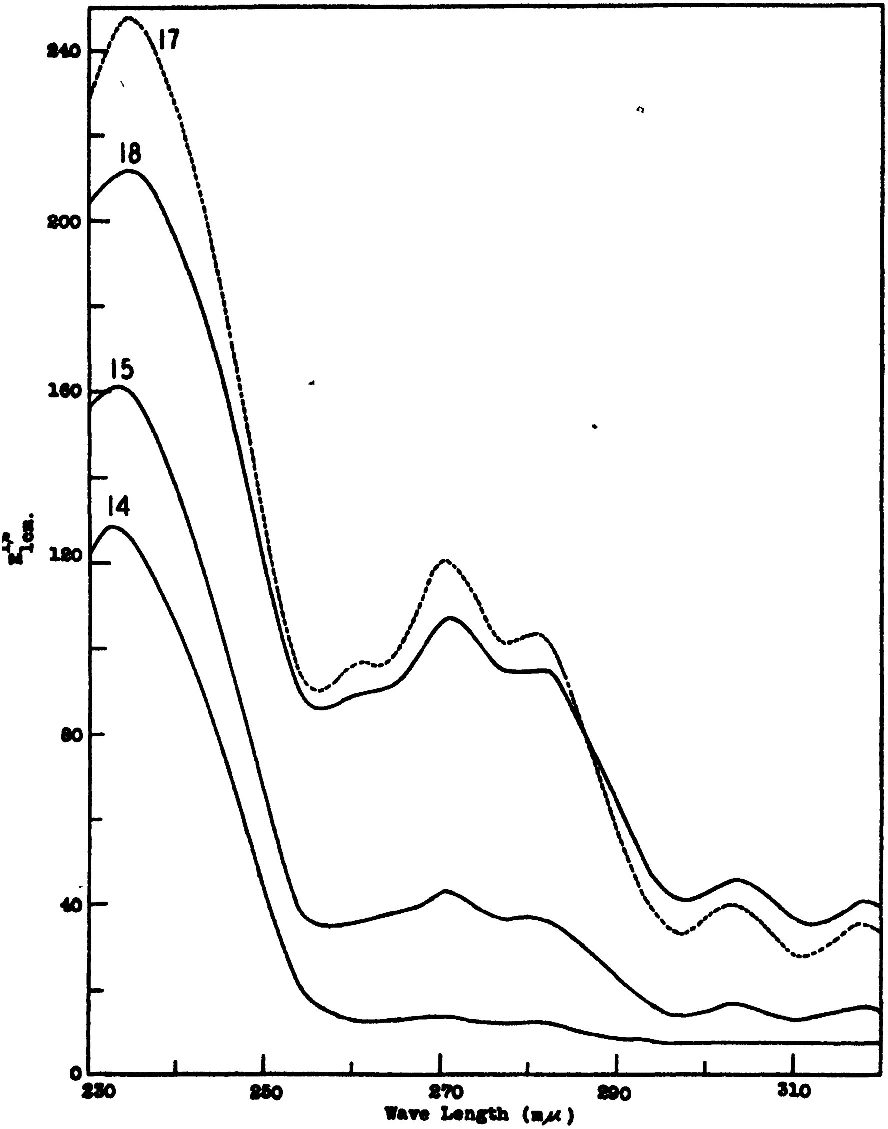

FIG. 2. Absorption curves of isomerized soaps, in aqueous solution, from ester Fractions 14, 15, 17, and 18 (mature human milk fat analysis).

material which would also contribute to the general absorption at 234 mμ is negligible. The presence of significant amounts of high molecular weight dienoic acids in the latter fractions of each distillation is further evidenced by the iodine values which are much higher than can be accounted for by the eicosatetraenoic acid alone.

The presence of hexade cenoic acid in the C_{16} fractions from the distillation of 3rd day colostrum was verified by the isolation of a product by fractional crystallization of the mixed acids of the C_{16} distillate fractions, which was mainly hexadecenoic acid as judged by iodine value and saponification equivalent.

Analyses—
Hexadecenoic acid. Calculated. I No. 99.0, saponification equivalent 254.4
Found. " " 88.9, " " 255.6

Characterization of the diethenoid C_{18} acid was attempted through the separation of characteristic bromides. No crystalline tetrabromostearic acid was obtained upon bromination of the free acids of the C_{18} fractions in petroleum ether. However, a heavy red liquid, insoluble in petroleum ether, was separated; bromine analysis indicated the presence of a mixture of di- and tetrabromostearic acids.

Analyses—$C_{18}H_{32}Br_4O_2$. Calculated. Br 53.26
$C_{18}H_{34}Br_2O_2$. " " 36.47
Found. " 49.62, 49.51

It appears, therefore, that the octadecadienoic acid in human milk fat may not be identical with linoleic acid of ordinary seed fat, although the latter may be present in traces.

DISCUSSION

The results of these analyses indicate several definite trends in the amount and composition of the human fat as the period of lactation progresses.

Fat as percentage of total solids in the milk increases markedly as the milk passes through the colostral stages and becomes mature milk. Likewise, the percentage of phospholipid (very high in 1st and 2nd day colostrum) decreases to a much lower value in later milk.

No significant changes in the proportion of low molecular weight acids appear during this transition from the first milk secretion to mature milk. However, there appears to be a regular increase in the amount of C_{12} and C_{14} acids and a very significant decrease in stearic acid and all the acids of greater molecular weight than C_{18}. These changes, a decrease in amount of higher acids and increase in amount of lower acids, may well be associated with the functional development of the lactating mammary gland following parturition. The remarkable constancy found for the percentages of oleic acid and the volatile acids is not in agreement with reports that colostral fat is richer in oleic acid (6) and contains less of the volatile fatty acids (7) than normal milk.

The possibility that different diets of the mothers are responsible for these changes is largely ruled out by the fact that all the milks analyzed were pooled samples. While it is true that the composition of most tissue fats depends to a large degree upon the kind of fat in the diet, it has been found that cow's milk fat is affected only slightly by variation in diet (5) unless the dietary fat is especially abundant in certain fatty acids (8). Even under the latter conditions, cow's milk fat is of fairly constant composition. If these experiences with cow's milk fat can be correlated with the human study, then the present analyses may be expected to be representative of the human milk fats obtained at various specific stages of lactation. Under special dietary conditions, slight variations might be expected.

In a comparison of these analyses with cow's milk fat (3), several differences in composition are apparent. The most obvious and interesting of these is the relatively small amounts of low molecular weight fatty acids in the human milk fats. Although this fact has been known previously, there has been a tendency to emphasize the occurrence of these acids in cow's milk fat as having some special significance in infant feeding. These acids, however, are not present in human milk fat in sufficient quantity to make the infant's need for them credible solely on the basis of their occurrence.

Another point of difference is the much larger amount of octadecenoic and octadecadienoic acids in human milk fat than in cow's milk fat. In fact, the molar percentage of total unsaturated acids is significantly greater for the former (about 50 per cent) than for the latter (about 35 per cent). It is of considerable interest, however, that the relatively high amount of octadecadienoic acid present in the human milk fat does not have the characteristics of the linoleic acid of ordinary seed fat. All attempts to obtain crystalline tetrabromostearic acid were unsuccessful. However, the liquid bromides, insoluble in petroleum ether or ethyl ether, obtained on bromination of the C_{18} fractions were mainly tetrabromostearic acid. Additional verification for the presence of appreciable amounts of octadecadienoic acid in the C_{18} fractions is furnished by the spectrophotometric analyses of the alkali-isomerized soaps of these fractions. The typical conjugated diene absorption peak at 234 mμ (for example, see Fraction 15 of Fig. 1) was shown for all fractions containing C_{18} esters, and the amount of octadecadienoic acid was calculated by comparing the amount of absorption at this peak with standard values obtained by isomerizing pure methyl linoleate.

Absorption peaks at 270 mμ, typical of isomerized trienoic acids, were also obtained and were calculated as octadecatrienoic acid by comparison with absorption values obtained after isomerization of pure methyl linolenate. However, since the amount was very small, it is possible although not probable, that this material could have come from the octadecadienoic acid. A small amount of triene material has been shown to appear during the distillation and manipulation of dienoic acids and their derivatives (9). The amount of eicosatetraenoic acid may also include other tetraenoic acids, as suggested by Bosworth. Since it was impossible to calculate accurately the amount of single dienoic acids of the C_{20} and C_{22} series, all the unsaturation not accounted for by the eicosatetraenoic acid was calculated as eicosadienoic acid. This calculation is not strictly justified, but since the actual amount is small, the over-all error is also small. The same is true for the calculation as eicosanoic acid of all the saturated acids having molecular weights greater than C_{18}.

The presence of other unsaturated acids, those containing 10, 12, 14, and 16 carbon atoms, was verified by the iodine values and saponification equivalents of the distillate fractions of the methyl ester. Unfortunately, the small amount of material available for the analyses precluded the possibility of isolating any but the hexadecenoic acid in degree of purity sufficient to confirm its presence. However, previous analyses of known mixtures[2] have shown that the fractional distillation procedure used in these analyses provides separation of the lower boiling fractions so as to prevent any contamination with C_{18} unsaturated acids.

SUMMARY

1. Total fat, phospholipid, and complete fatty acid analyses of composites of the fats of human colostrum of the 1st and 2nd days, human colostrum of the 3rd day, and mature human milk were determined.

2. The percentage of total fat increases and the phospholipid decreases as the lactation period progresses.

3. The amount of low molecular weight fatty acids is very small as compared to cow's milk fat analyzed in an identical manner.

4. There is a relatively large amount of C_{20} and C_{22} acids in the colostral fats, and these acids decrease while the C_{12} and C_{14} acids increase as the postpartum period lengthens.

The authors are grateful for the collaborative assistance of Dr. Icie Macy Hoobler and Dr. Harold H. Williams in securing and drying the milk samples used in this study, and for additional financial assistance provided by The Buhl Foundation.

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REJUVENATION OF PHOSPHATE IN ADENINE NUCLEOTIDES

I. ENZYMATIC METHODS FOR SEPARATION OF PHOSPHATE GROUPS IN POLYPHOSPHORYLATED NUCLEOTIDES*

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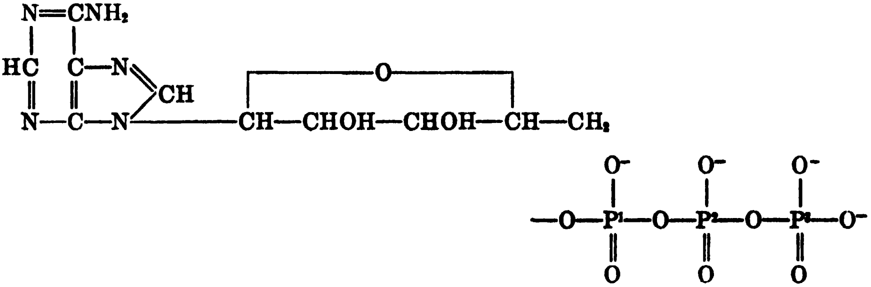
(Received for publication, March 17, 1944)

Adenosine triphosphate has been shown by a large number of investigators to be of importance in the transfer of phosphate. The tri- or diphospho compounds liberate or donate the labile phosphate groups, whereas the dephosphorylated compound, adenylic acid, accepts phosphate from phosphoamidines (1, 2), phosphoenol pyruvate (3), or phosphocarboxylates (4, 5). A study of trans- and dephosphorylations in the intact animal became possible after the introduction of isotopic phosphate into biological studies (Hevesy (6)). The aim of the present paper has been to find methods which would permit a separate isotope analysis of the two labile phosphate groups in adenosine triphosphate.

The phosphate groups in adenosine triphosphate have been numbered as illustrated in the accompanying formula.



Phosphate group 1 is stable; *i.e.*, it does not participate in the rapid trans- and dephosphorylations which involve the phosphate groups 2 and 3. It has been found that a number of enzymatic trans- and dephosphorylations *in vitro* involve only the third (terminal) phosphate group.

The following enzymes have been shown to catalyze reactions involving the third phosphate group exclusively (one-step transphosphorylases[1]).

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* Aided by a grant from the Commonwealth Fund.

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[1] An enzyme which catalyzes the transfer of phosphate from one organic linkage to another.

Dephosphorylations		
Adenosine triphosphatase	Lobster muscle	(2)[*]
" "	Myosin from rabbit muscle	(7)
Inosine triphosphatase	" and muscle extract from rabbit muscle	(8)
Transphosphorylations		
Hexokinase	Bakers' yeast	(9)
Arginine transphosphorylase[†]	Lobster muscle	(2)
Phosphoglyceric acid transphosphorylase	Brewers' and bakers' yeast	(4, 10)
	Rabbit muscle	(10)

However, in other enzyme systems trans- and dephosphorylations of adenosine triphosphate involve both labile phosphate groups (two-step transphosphorylations).

Dephosphorylations		
Adenylpyrophosphatase	Liver	(11)
"	Potato	(12)
Inosine triphosphatase	"	(12)
Transphosphorylations		
Acetyl phosphate transphorylase		(13)
Creatine transphosphorylase	Rabbit muscle	(1)
Phosphopyruvate transphosphorylase	" "	(3, 14)

The phosphate group 1 is split off by a specific enzyme, the so called 5-nucleotidase (15). This phosphate group participates under certain circumstances in the phosphate cycle (16, 17).

Studies of the rate of rejuvenation of phosphate groups in intact animals with radioactive phosphate (P^{32}) have revealed a rapid shift of phosphate in the pyrophosphate part (phosphate groups 2 and 3) of adenosine triphosphate, whereas the first phosphate was shown to be rejuvenated at a much slower rate (Korzybski and Parnas (18); Hevesy (19)). Korzybski and Parnas showed that within 30 minutes after the injection of radioactive phosphate the muscle pyrophosphate has reached the same isotope concentration as that of the inorganic phosphate in the muscle.

Meyerhof *et al.* (20) showed a rapid rejuvenation of the pyrophosphate group *in vitro.*

In the present studies an attempt has been made to obtain an estimate of the rates of rejuvenation of the second and third phosphate groups in resting muscle and liver and, in particular, to compare the rates of rejuvenation of the two phosphate groups.

The separation of phosphate groups 2 and 3 is not possible by any ordinary chemical procedure. However, any one of the enzyme systems listed above as one-step transphosphorylases can be used as a tool in a separate isotope determination of the second and the third phosphate groups. Of these enzymes, hexokinase from bakers' yeast has been used most extensively in the present work. This enzyme, which can be obtained as a relatively stable preparation which remains active for several months, is not only strictly specific towards adenosine triphosphate, but is completely free of any other enzyme which could mobilize the second phosphate group (21).

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[*] Bibliographic reference numbers.

[†] See foot-note 1.

Isotope Analysis of Phosphate Groups in Adenosine Triphosphate—Adenosine triphosphate is isolated according to Lohmann (22) from the muscles of an animal which has been injected with radioactive phosphate a few minutes previously. The isotope concentration of the complete nucleotide is determined.[2] In order to separate the stable phosphate group (No. 1) from the two labile groups (phosphates 2 and 3), the nucleotide is subjected to hydrolysis with barium hydroxide at 100° (22). This gives rise to a liberation of inorganic pyrophosphate (phosphates 2 and 3) and adenylic acid (phosphate 1). A separation between the stable and labile phosphate groups can also be effected by adding potato adenylpyrophosphatase (12) to adenosine triphosphate. In this case, the second and third phosphate groups are obtained as orthophosphate. The values obtained by the two methods are in agreement (see Paper II, Table VII). The latter method has also been used in order to separate the first and second phosphate groups in adenosine diphosphate. In this case the hydrolysis with barium hydroxide is very slow.

In order to separate the second and third phosphate groups, adenosine triphosphate is incubated with glucose in the presence of yeast hexokinase. When the phosphate transfer has proceeded to completion, adenosine diphosphate and hexose monophosphate (6-phosphohexose) are isolated through the barium and mercury salts (see the details later).

The results of isotope analysis of the three phosphate groups in adenosine triphosphate from rabbit and frog muscle will be presented in Paper II (*cf.* (23), Tables V and VI).

Isotope Analysis of Hexose Monophosphate—In order to analyze hexose monophosphate present in muscle extract or formed in the hexokinase test, it is sufficient to precipitate the ester either with barium acetate and ethanol or with lead acetate,[3] remove traces of adenine nucleotides with mercuric salts in acid medium, and finally precipitate the ester with calcium acetate and hot ethanol, thus obtaining the crystalline calcium hexose monophosphate (24).

In the presence of glycerophosphate it is not possible to obtain pure hexose monophosphate owing to formation of a double salt between glycerophosphate and hexose monophosphate (Smythe (25)). A very specific method for the separation of hexose monophosphate P in the presence of glycerophosphate is the enzymatic conversion of glucose-6-phosphate to glucose-1-phosphate by the enzyme phosphoglucomutase (26). Colowick and Sutherland (27) found that 4.5 to 5 per cent of the glucose-6-phosphate is converted to glucose-1-phosphate. The phosphorus of the latter compound can be liberated by 5 minutes hydrolysis in boiling N sulfuric acid or enzymatically by conversion to starch by phosphorylase (28) and analyzed for isotopic phosphorus. This method furthermore makes it possible to estimate what percentage of the phosphorus in a fraction is hexose monophosphate (see the experimental section).

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[2] The details of the isotope analysis will be described in a subsequent paper (23).

[3] When lead acetate was used, the tissues were always fixed with trichloroacetic acid and all metals used were added as acetates.

It thus appears that a number of enzymatic reactions are of value as tools for isotope analyses of several important phosphate compounds. There is reason to believe that enzymatic methods might also prove to be useful in isotope analyses of other elements.

EXPERIMENTAL

Isotope Analyses of Adenosine Triphosphate—75 gm. of frog muscle were minced and extracted twice with a solution of 2.5 per cent $HgCl_2$ in 0.5 N HCl. The filtrate was treated with H_2S gas, the aerated filtrate was made alkaline to pH 8, and barium acetate was added. The barium precipitate, which contains the adenosine triphosphate and the inorganic phosphate, was washed and dissolved in dilute HNO_3. The adenine nucleotide was precipitated with mercuric nitrate, washed, freed of Hg with H_2S, and the aerated filtrate was neutralized and analyzed.

Total amount of organic P, 13.8 mg., corresponding to approximately 140 mg. of Ba salt. The ratio of labile to total P was 0.64; calculated, 0.67.

Adenosine triphosphate in an amount corresponding to 6.4 mg. of labile P (or 9.6 mg. of total P) was incubated at 30° and at pH 7.2 with 100 γ of hexokinase from yeast in the presence of 3 mg. of $MgCl_2$ and 60 mg. of glucose. After 1 hour the protein was precipitated with trichloroacetic acid and the *adenosine diphosphate* (phosphate groups 1 and 2) formed was precipitated with barium at alkaline reaction, redissolved in HNO_3, precipitated with mercuric nitrate, decomposed with H_2S, and neutralized. The total organic P thus recovered from the barium precipitate equals 5.3 mg. of P; maximum yield 6.8 mg. of total P., *i.e.* 80 per cent yield.

A sample of this adenosine diphosphate containing 515 γ of P was digested and analyzed for N. Found, 545 γ of N; calculated, 580 γ of organic N; the N:P ratio was, therefore, found to be 1.06, the theoretical ratio for adenosine diphosphate being 1.12. The ratio of labile to total P was 0.50; calculated, 0.50.

The third phosphate group which was esterified to hexose was recovered from the supernatant fluid after the barium precipitation of adenosine diphosphate. The barium hexose monophosphate was precipitated by addition of an equal volume of ethanol and redissolved in acid. Traces of adenine nucleotide were removed by precipitation with mercuric acetate. The hexose monophosphate which contains the third phosphate group remains in the supernatant. The total organic P of this fraction was 1.8 mg. of P; maximum yield, 3 mg. of P, *i.e.* 60 per cent yield. In order to identify the material, the ester was precipitated as the crystalline calcium salt by adding calcium acetate and an equal volume of ethanol to a solution of the ester; the mixture was heated to 70°, which gives rise to formation of a crystalline calcium precipitate.

An amount of this calcium precipitate containing 500 γ of organic P consumed 15.5 micromoles of I_2 in the Macleod-Robison titration, corresponding to 2800 γ of glucose, or since approximately one-fourth of the hexose of the ester is fructose, 3500 γ of total hexose. An amount of 500 γ of organic P would correspond to approximately 3000 γ of total hexose.

Isolation of Adenylic Acid—A portion of the adenosine *di*phosphate fraction containing 1100 γ of labile P was incubated with 150 γ of potato adenylpyrophosphatase and 1 mg. of $CaCl_2$; pH 6.5, temperature 30°, incubation time 30 minutes. The orthophosphate liberated was precipitated as the barium salt. The total amount of orthophosphate was 890 γ of P. The barium salt of adenylic acid which remained in the supernatant fluid was precipitated by addition of 1.5 volumes of ethanol. The barium precipitate was dissolved in water, and the solution brought to about pH 3 and precipitated with mercuric nitrate. The mercury salt was washed twice with water, decomposed with H_2S gas, and the aerated filtrate which contained the free acid was analyzed.

The total amount of organic phosphate in this fraction was 510 γ of P; the maximum yield corresponded to 1100 γ of P, *i.e.* approximately 50 per cent yield. No inorganic P was found, and only 1.3 per cent of the organic P was labile. A sample containing 77 γ of P was digested and analyzed for nitrogen. Found, 180 γ of N; calculated, 174 γ of N for adenylic acid.

Enzymatic Analysis of Crude Hexose Monophosphate—Adenosine triphosphate containing 2850 γ of labile P was incubated with 67 γ of purified yeast hexokinase, 50 mg. of glucose, and 2 mg. of $MgCl_2$; pH 7.2, temperature 30°. After 30 minutes incubation, the adenosine *di*phosphate formed was precipitated with barium acetate. Yield, 835 γ of labile P, which is 60 per cent of the maximum amount.

The remaining organic pyrophosphate in the supernatant fluid was hydrolyzed by boiling in dilute HCl. The inorganic phosphate liberated was removed by precipitation with barium hydroxide. The supernatant fluid yielded 1240 γ of organic P, containing 200 γ of acid-labile P before hydrolysis or 840 γ of organic P after hydrolysis. The volume of the barium supernatant was 3.3 ml.; 1.5 ml., containing 380 γ of organic P, were incubated with 600 γ of phosphoglucomutase,[4] 2 mg. of glutathione, and 2 mg. of $MgCl_2$. The control contained the same components except the enzyme.

In the presence of enzyme, 8.9 γ of acid-labile P (P split after 5 minutes boiling in HCl) was formed, corresponding to 198 γ of glucose monophosphate P (4.5 per cent glucose-1-phosphate to 95.5 per cent glucose-6-phosphate (28)) or 436 γ in the whole sample. Since the whole sample contained 840 γ of organic P, the percentage of P due to glucose monophosphate is $436/840 \times 100 = 52$ per cent or with one-third to one-fourth as fructose-6-phosphate; the percentage of hexose monophosphate was approximately $570/840 \times 100 = 70$ per cent. The chief impurity in the hexose monophosphate fraction after acid hydrolysis is probably ribose-5-phosphate.

SUMMARY

An enzymatic method by which the two labile phosphate groups in adenosine triphosphate can be separated has been described. The terminal phosphate group was transferred to glucose by the enzyme hexokinase. The second phosphate group was liberated by the addition of an adenylpyrophosphatase preparation from potato. The specific enzymatic separation of the phosphate groups in adenosine triphosphate makes possible an analysis of isotopic phosphate in each of the phosphate groups of the nucleotides.

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REJUVENATION OF PHOSPHATE IN ADENINE NUCLEOTIDES

II. THE RATE OF REJUVENATION OF LABILE PHOSPHATE COMPOUNDS IN MUSCLE AND LIVER*

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(Received for publication, March 17, 1944)

The present studies deal with the uptake of phosphate in muscle and with the rate of rejuvenation of phosphate in phosphocreatine and in the pyrophosphate of adenosine polyphosphates.

These studies of phosphorus metabolism by means of radioactive phosphorus had three main purposes: (1) to obtain information about the nature of phosphate uptake in the muscle; (2) to obtain an estimate of the rate of rejuvenation of the labile phosphate groups in resting muscle; (3) to measure individually the rates of rejuvenation of the two labile phosphate groups in adenosine triphosphate (phosphate groups 2 and 3) and to compare them with that of phosphocreatine.

Before these problems could be solved, however, it was necessary to find a method for determining the intracellular inorganic phosphate without interference from the extracellular highly radioactive phosphate.

Hevesy and Rebbe (1) showed that phosphate penetrates the skeletal muscle very slowly and as a consequence the specific radioactivity (radioactivity per mg. of P) of the extracellular phosphate remains for a long time much higher than that of the intracellular phosphate.

It is obvious that failure to take into account the existence of such an extracellular pool of highly radioactive phosphate would give rise to excessively high values for the P^{32} concentration in the inorganic fraction in muscle as compared with that of the organic fractions.

On that account the interpretations of Sacks in 1940 (2) and of Bollman and Flock in 1943 (3) with regard to rejuvenation of phosphocreatine and adenyl pyrophosphate in muscle cannot be considered valid, since no corrections were made for the extracellular phosphate. Sacks and Altschuler in 1942 (4) made corrections for the extracellular phosphate. However, since they did not measure the extracellular space but used an average value, these calculations are necessarily crude and do not constitute a basis for interpretations. Furchgott and Shorr (5) have recently obtained direct

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* This work was supported by a grant from the Commonwealth Fund.

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values for P^{32} concentration of extra- and intracellular phosphate in slices of heart muscle equilibrated *in vitro* in a medium containing P^{32}.

The errors made by identifying the inorganic P obtained by direct extraction of a muscle with that of the intracellular inorganic P are fairly small with respect to analytical values (mg. per cent of P) but are large with respect to specific radioactivity. Even larger errors may appear when small animals, such as rats, are injected with radioactive P containing as much as 7.5 mg. of P (*cf.* Bollman and Flock (3)), thus raising considerably the level of inorganic P in serum; under such conditions the P^{32} concentration of the combined extra- and intracellular phosphate can reach values as high as 15 times that of the P^{32} concentration of the intracellular phosphate alone. These considerations may also apply to the results obtained by the continuous administration of P^{32} (6).

In order to estimate the errors due to inclusion of extracellular P, extracts from unperfused muscle and from muscle perfused with ice-cold Ringer's solution were compared with respect to specific radioactivity of the inorganic P. The specific activities of serum and of successive portions of the perfusion fluid were also determined. Since the experiment was performed in order to study the rejuvenation of labile P groups, the time from the injection of P^{32} (intravenous) to the fixing of the muscles did not exceed 30 minutes. The details of the experiment will be described in the experimental section (Protocol 1).

TABLE I

P^{32} Concentration of Inorganic Phosphate from Unperfused and from Perfused Skeletal Muscle (Cf. Protocol 1)

	Relative specific activity
	per cent
Serum inorganic P	6650
Inorganic P in unperfused muscle	235 (135% error)
" " " perfused muscle	100

The error due to extracellular P in the unperfused leg can also be calculated if the extracellular and serum inorganic phosphates are assumed equal in concentration and radioactivity. Assuming that the extracellular space is equal to 12 per cent of the muscle (7) and that the serum phosphate concentration is one-fourth to one-fifth that of the muscle phosphate, then since the serum P in Table I is about 66 times more active than muscle P, the error would be $(0.12 \times 0.20 \times 66) \times 100 = 158$ per cent, which agrees with the 135 per cent increase actually found.

The error due to extracellular P remaining in the perfused leg can be calculated, since different portions of the perfusion fluid were tested for radioactivity. If the radioactivity per ml. of serum is considered 100, the P^{32} concentrations in various portions of the perfusion fluid were as described in Table II.

The maximal error is, therefore, $(0.12 \times 0.20 \times 0.04 \times 66) = 6$ per cent. In the estimation of the specific activity of intramuscular inorganic P there is another source of error to be kept in mind. During the period of perfusion of the leg (with occlusion of the other leg) phosphocreatine breaks down in both legs. The analytical amount of inorganic P increases, whereas the specific activity decreases, since it is diluted with phosphate from a large pool, the phosphocreatine pool, which has, at least initially, a lower specific activity than the inorganic phosphate.

TABLE II

P^{32} Concentration in Serum and in Perfusion Fluid of Muscle (Cf. Protocol 1)

	Relative P^{32} concentration per ml.
	per cent inorganic P activity
Serum	100
Middle 400 ml. perfusion fluid	8
Last 400 ml. perfusion fluid	4

TABLE III

P^{32} Concentrations in Phosphate Fractions from Muscle (Cf. Protocol 1)

	Radioactivity, per cent of inorganic P	
	Perfused muscles	Unperfused muscles
Inorganic P	100	235
Phosphocreatine P*	61	61
Pyrophosphate in small sample	56	58
Labile P of isolated adenosine triphosphate	57	58
Stable " " " " "	8	

* See foot-note 1.

In the experiment in Table III the inorganic P has been diluted with about an equal amount of P from the phosphocreatine pool, which has a somewhat lower specific activity. The specific activity of the original inorganic P might therefore be as high as 120 to 130 instead of 100, which would make the specific activity of the organic labile phosphate less than 50 per cent of that of the inorganic phosphate. It is important to emphasize that the contribution of phosphocreatine P to the pool of inorganic P was the same on the perfused and the unperfused side.[1]

The lower isotope concentration in the labile P esters compared with inorganic P has a direct bearing on the question, does phosphate enter the muscle exclusively by a metabolic reaction (for instance a reaction between phosphate and glycogen or aldehyde groups) or does phosphate enter the muscle primarily by exchange? The problem is of importance too for the understanding of the mechanism of absorption of phosphate and of glucose in the intestine and the kidney tubules. According to Sacks and Altschuler (4), phosphate penetrates the muscle exclusively by breakdown and resynthesis of phosphocreatine and adenyl pyrophosphate.[2] In their studies on skeletal muscle the isotope concentration of the labile P esters was always found by these workers to be as high as the inorganic P. In heart muscle Sacks and Altschuler in some cases found higher isotope concentrations in the labile esters than in the inorganic P. Their calculations of the extracellular and intracellular phosphate, were, however, too crude to permit such far reaching conclusions. Moreover, Furchgott and Shorr (5) in experiments with heart muscle *in vitro* showed that the P^{32} concentration of the labile phosphate groups is markedly lower than that of the intracellular phosphate, in particular at low temperature. The authors emphasize that these observations are hardly compatible with the hypothesis of formation of an organic labile phosphate ester intermediate in the passage of phosphate into the cell.

The present experiments are in agreement with the findings of Furchgott and Shorr (*cf.* Table IV).

Provided that the ammonium-magnesium precipitate represents only inorganic phosphate, it seems certain that phosphate enters the muscle primarily by a physical exchange. The possibility that phosphorolysis of glycogen plays a rôle in the uptake of phosphate is discussed later in this paper.

The rate of penetration of P into the skeletal muscle can be calculated from the data of the relative P^{32} concentrations of the extra- and intracellular phosphate and from the concentration of inorganic phosphate in the muscle. Resting skeletal muscle contains approximately 250 γ of P per gm. The P^{32} concentration of the muscle phosphate 20 minutes after the injection of P^{32} was only 1 to 2 per cent of that of the extracellular phosphate. The P^{32} concentration of plasma was identified with that of the extracellular, an assumption which does not hold true within the first 5 minutes when the extracellular phosphate has not yet come to an equilibrium with the plasma. If we estimate the ratio between the P^{32} concentrations of the extra- and intracellular phosphates after 20 minutes as 50:1, it means that only 2 per cent of the muscle phosphate or 5 γ of P have been rejuvenated in that time. The rate of penetration of phosphate per gm. of muscle per minute would therefore amount to approximately 0.25 γ of P. The intracellular phosphate is, however, a part of the metabolic wheel of the cell and in a living cell it is continuously drawn upon and renewed from the labile phosphoric esters (phosphocreatine, adenyl pyrophosphate, and to some extent hexose monophosphate). The rate of penetration is consequently 3 to 5 times higher than that calculated on the basis of a "dead" pool of inorganic phosphate. A rate of penetration of approximately 1 γ of P per minute per gm. of muscle would be a fair estimate.

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[1] The specific activity of the organic labile phosphate in the experiment was probably somewhat lower than 50 per cent of the original inorganic P if corrected for phosphocreatine P breakdown.

[2] "... phosphate enters or leaves the cell only by formation or breakdown of an organic compound at the membrane" (Sacks and Altschuler, 1942 (4); *cf.* also (8)). In 1943 Sacks (9) wrote, as a conclusion of his experiments, "that inorganic phosphate enters into the intracellular inorganic phosphate only by hydrolysis of PC or ATP formed at the membrane."

TABLE IV

Rejuvenation of Labile Phosphate Groups in Skeletal Muscle (Cf. Protocol 1)

The results are expressed as per cent of P^{32} concentration of inorganic P in muscle.

Animal	Type of injection of P^{32}	Time after injection	Inorganic P		Phosphocreatine P		2,3-Pyrophosphate P	
			Unperfused muscle	Perfused muscle	Unperfused muscle	Perfused muscle	Unperfused muscle	Perfused muscle
		min.						
Bullfrog	Intravenous	20		100		10		26
"	Subcutaneous	20		100		22		20
Rabbit	Intraperitoneal	30		100			59	54
"	Intravenous	30	235	100	61	61	58	57
"	Intraperitoneal	180	160	100		100	94	94

The rate of rejuvenation of pyrophosphate can also be calculated from the data obtained. Mammalian muscle contains between 0.2 and 0.3 mg. of pyrophosphate P per gm. Approximately half of this fraction is renewed from the pool of inorganic phosphate within 20 minutes (Tables IV and V). This means a rate of 5 γ of P per minute. However, the phosphocreatine pool, which was found to have the same P^{32} concentration as that of the adenyl pyrophosphate pool, is rejuvenated through the latter pool. The rate of rejuvenation of the pyrophosphate pool ought to be calculated on the basis of the combined pools of pyrophosphate and phosphocreatine, since the latter and adenyl pyrophosphate are in an enzymatic equilibrium (10), which is rapidly attained.

The phosphocreatine pool amounts to between 0.55 and 0.7 mg. of P per gm. of muscle and the combined pools amount therefore to 1 mg. of P. Half of this amount is rejuvenated in 20 minutes, which means *a rate of rejuvenation of 25 γ of P per minute and per gm. of resting rabbit muscle.*

Isotope Analysis of Hexose Monophosphate Fraction in Muscle—A limited number of P^{32} analyses of hexose monophosphate in muscle were made. The isotope concentration as compared with the labile phosphate groups varied a great deal. In some experiments the hexose monophosphate P had the same P^{32} concentration as that of phosphocreatine and adenyl pyrophosphate; in other cases it was either lower or higher (*cf.* Table V).

TABLE V

Comparison of P^{32} Concentrations in Hexose Monophosphate of Muscle and Labile Phosphate Groups of Adenosine Triphosphate

Animal	Temperature	Time after injection of P^{32}	Activity, per cent of inorganic P	
	°C.	*min.*		
Rabbit......	37	15	Hexose monophosphate	67*
			Adenosine triphosphate†	50
"	37	20	Hexose monophosphate	40
			Adenosine triphosphate†	60
" ...	37	180	Hexose monophosphate	220*
			Adenosine triphosphate†	93
"	37	360	Hexose monophosphate	45
			Adenosine triphosphate†	95
Frog.......	20	20	Hexose monophosphate	20
			Adenosine triphosphate†	56
" ...	20	20	Hexose monophosphate	14
			Adenosine triphosphate†	55
" ..	5	20	Hexose monophosphate	0.5
			Adenosine triphosphate†	22

* Washed with inert phosphate.

† Labile phosphate.

The very high value of the hexose monophosphate P in the 3 hour experiment is particularly remarkable, because the P^{32} concentration considerably exceeds that of the intracellular inorganic phosphate. The relation to the glycogen content of the muscle was not studied. This latter question, however, may be of significance in explaining these variations (see "Discussion").

Fractional Isotope Analysis of Adenosine Triphosphate—Korzybski and Parnas (11) showed that the stable phosphate in adenosine triphosphate has a far lower isotope concentration than the two labile phosphates, which were determined together as pyrophosphate. Meyerhof *et al.* (12)

demonstrated a very rapid rejuvenation of the pyrophosphate group in isolated enzyme systems. In the present paper the two labile groups were determined separately by a stepwise enzymatic cleavage described in Paper I (13).[3] Table VI furnishes an example of a complete isotope analysis of adenosine triphosphate from resting rabbit muscle 20 minutes after the intravenous injection of radioactive phosphate.

Table VI shows that even in a resting muscle both labile phosphates are involved in the rapid breakdown and resynthesis of adenosine triphosphate. This is to be expected, since skeletal muscle contains an enzyme, myokinase (15), which catalyzes the exchange of the second and third phosphate groups (16).

Rejuvenation of the Labile Phosphate Compounds in Frog Muscle—Experiments performed on bullfrogs at a low temperature (0–5°) indicated that the exchange between the two labile groups of adenosine triphosphate is very incomplete. Such experiments are illustrated in Table VII.

TABLE VI

P^{32} Concentration 20 Minutes after Injection of Radioactive Phosphate in Various Phosphate Groups of Adenosine Triphosphate from Rabbit Muscle (Cf. Protocol 1)

Phosphate group No	P^{32} (specific activity), per cent of inorganic P activity
2, 3	57
2, 3. After "washing" with inert phosphate	56
2	61
3	53*
1	8

* The slightly lower isotope concentration of the third phosphate is due to contamination with a small amount of ribose phosphate (phosphate 1).

A bullfrog was injected intravenously with 50 microcuries of P^{32}. The hind legs were kept at a temperature of 20° and perfused with Ringer's solution for 20 to 30 minutes before fixing (*cf.* Protocol 3).

It will be noticed that in these experiments the third phosphate group has a lower P^{32} than the second. This phenomenon has been found in a number of other frog experiments, but only in those cases in which the P^{32} concentration of phosphocreatine was markedly lower than that of the pyrophosphates (Experiments 1 and 2). Mincing the muscle gives rise to a marked splitting of phosphocreatine, the phosphate of which must have been transferred preferentially through position 3 in the adenosine triphosphate before being liberated.

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[3] Furchgott and Shorr (5, 14) have recently used a similar enzymatic cleavage. The terminal group was split off by additions of adenosine triphosphatase from washed lobster muscle. Direct P^{32} measurements were only made on the second phosphate group and the P^{32} concentration of the third calculated.

It has already been mentioned that an analysis of frog muscle at low temperatures shows that the hexose monophosphate in the skeletal muscle had attained only a very low concentration of P^{32} (*cf.* Table V).[4]

It appears as if some of the enzymes converting glycogen and phosphate to hexose monophosphate were inactive in the frog muscle at the low temperature. One of these enzymes, the phosphoglucomutase (17) is active only when reduced (18). Moreover, it is known (19) that frogs kept at low temperature develop very little lactic acid and do not use their glycogen even when stimulated to exhaustion.

TABLE VII

P^{32} Concentrations in Phosphocreatine and Adenosine Triphosphate from Frog Muscle (Cf. Protocol 3)

The frogs were held at a temperature of about 20° in Experiments 1, 2, and 4; in Experiment 3, at 5°.

	P^{32}, per cent of inorganic P activity			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Phosphocreatine P	10			22
Adenosine triphosphate, groups 2 and 3.	25	28	22	20
" " group 2.	38	41	27	19
			26.5*	
" " " 3 (terminal)	9	11	9	20
" " " 1	0.5–1		1	

* Phosphate group 2 liberated as orthophosphate by hydrolysis of the adenosine diphosphate with potato adenylpyrophosphatase (*cf.* (13)).

Rejuvenation of Phosphate in Liver—The rate of rejuvenation of phosphorus compounds in the liver has been estimated in some experiments by Lundsgaard (20). Analyses of the P^{32} concentrations of the inorganic phosphate in plasma and liver and of the acid-labile phosphoric esters in the liver were made as early as 30 minutes after the injection of radioactive phosphate. The two outstanding features of Lundsgaard's studies were (1) the rapid penetration of phosphate into the liver cells; (2) the rapid rate of rejuvenation of the acid-labile phosphoric esters (including adenyl pyrophosphate).

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[4] The hexose monophosphate was purified and identified by aldose determination. Moreover, the muscle contained lactic acid, which in view of the lack of isotopic phosphate in the hexose phosphate could not have been formed from glycogen and inorganic phosphate, but must have been formed directly at the expense of the hexose monophosphate; the latter was actually found to be lower than normal.

The relative P^{32} concentrations 30 minutes after the injection of the radioactive phosphate were 100 for plasma P, 41 for liver inorganic P, and 32 for liver acid-labile P. This means that the acid-labile P 30 minutes after the injection has 75 per cent of the P^{32} concentration of the inorganic phosphate in the liver. This is probably a minimum value, since the fraction called liver inorganic P includes some extracellular phosphate of higher radioactivity. The correction for extracellular phosphate in liver is small, however, because of the rapid penetration of phosphate into the liver cells.

The present studies of phosphate turnover in liver show that adenyl pyrophosphate is rejuvenated extremely rapidly. Rabbits were injected intravenously with 1000 to 1500 microcuries of P^{32} and after 5 minutes a few ml. of blood were collected and the liver removed, chilled, perfused with cold Ringer's solution, minced in the blender, and fixed with acid (see Protocol 4). Adenine nucleotides were isolated in the usual way; the material obtained was mainly adenosine diphosphate.

TABLE VIII

P^{32} Concentrations of Phosphate Fractions from Rabbit Liver

Rabbit liver, perfused with cold Ringer's solution 5 minutes after intravenous injection of radioactive phosphate (see Protocol 3).

	P^{32}, per cent of inorganic P activity	
	Experiment 1	Experiment 2
Serum, inorganic P	270	1200
Liver, perfused. Inorganic P (small sample)	100	100
" " Pyrophosphate P (small sample)	70	
" " ATP, ADP, pure compounds	83	82
" " 3rd phosphate group of ATP		80

Isotope analyses showed that within 5 minutes after the injection of P^{32} the pyrophosphate of the adenine nucleotide had reached almost the same P^{32} concentration as the intracellular inorganic phosphate (Table VIII). The rapid saturation of the liver pyrophosphate with P^{32} is, to some extent at least, attributable to the absence of a phosphocreatine pool and to the much smaller pool of pyrophosphate in liver compared with muscle. The actual rate of rejuvenation of pyrophosphate P amounts to 15 to 20 γ per minute per gm. of liver. This is, however, a minimum estimate, since the P^{32} concentration of the pyrophosphate was as high as 80 per cent of that of the intracellular inorganic phosphate.

DISCUSSION

It appears from the present studies that the intracellular inorganic phosphate of skeletal muscle is a fraction which is particularly difficult to separate from other fractions well enough to obtain reliable values for the P^{32} concentration. It was emphasized in the introduction that the presence of a pool of extracellular phosphate having a P^{32} concentration many times higher than that of the intracellular inorganic phosphate makes it urgently necessary either to remove the extracellular phosphate by perfusion or to try to calculate how much of the inorganic P^{32} is extracellular and how much is intracellular before any conclusions can be drawn. If the concentration as well as the specific radioactivity (P^{32} concentration) of phosphate in plasma is known, and the extracellular space in the muscle is either determined or an average value, for instance 12 per cent, is used, it is possible from the specific radioactivity of the combined extra- and intracellular phosphate to calculate the specific radioactivity of the intracellular phosphate.

Sacks in his publication of 1940 (2) does not give values for the phosphate concentration in serum nor for the P^{32} concentration in that fraction. It is therefore not possible to know how large a part of the P^{32} in the total inorganic phosphate belongs to the extracellular and how large a part to the intracellular phosphate. The studies reported by Sacks and Altschuler in 1942 as well as that reported by Bollman and Flock (3) furnish sufficient data to permit a calculation of the P^{32} concentration of the intracellular phosphate. Sacks and Altschuler calculated that the P^{32} concentration of plasma was 30 to 50 times higher than that of the total inorganic phosphate in muscle. The phosphate concentration in plasma was not stated.

The results of Bollman and Flock's studies (3), so similar to those of Sacks in 1940, may be interpreted in a similar manner. Rats weighing around 250 gm. were injected with isotopic phosphate containing as much as 1.5 to 7.5 mg. of P and the plasma phosphate must therefore have been raised considerably.

The relation between the phosphate in the whole muscle and in the muscle fibers can be expressed by the equation, mg. of P of total inorganic P × P^{32} concentration of total inorganic P = mg. of extracellular P × P^{32} concentration of plasma inorganic P + mg. of intracellular P × P^{32} concentration of intracellular inorganic P.[5]

Let the P^{32} concentration in the whole muscle be 100 and let the P^{32} concentration in plasma and extracellular space be 10-fold that of the muscle (*i.e.*, 1000). The extracellular space is considered to be 10 per cent, which is a minimum estimate. Inorganic phosphate in resting muscle, including the extracellular space, does not usually exceed 25 mg. per cent; the latter value corresponds to 27 mg. per cent of phosphate in the muscle fibers (intracellular phosphate). If the plasma phosphate is raised from 5 to 25 mg. per cent, the phosphate in the total muscle (intra- and extracellular phosphate) would be 27 mg. per cent. Since the extracellular space was assumed to be approximately 10 per cent of the muscle and the P^{32} concentration in the extracellular fluid was considered to be 10 times that of the combined inorganic phosphate of the muscle (*i.e.*, 1000 per cent), we have $27 \times 100 = 25 \times 1000 \times 0.10 + 27 \times X \times 0.9$ or $2700 = 2500 + 27.0 \times X$. $X = 200/27 = 7.4$; therefore, the P^{32} in the muscle fiber is only approximately 7 per cent of that in the directly estimated phosphate. Since Bollman and Flock in 1943 as well as Sacks in 1940 found the phosphocreatine P and pyrophosphate P to have isotope concentrations between 7 and 20 per cent of that of the directly determined inorganic phosphate, it means that the phosphoric esters probably already had acquired approximately the same isotopic concentration as the phosphate. It is therefore not surprising that all investigators have, so far, been unable to find a further increase in the isotope concentration of pyrophosphate in the contracting muscle. The experimental basis for Sacks' statement (2) that "... the results show that adenosine triphosphate does not act as phosphate transporter in the formation of lactic acid ... " is very doubtful.

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[5] At the end of the experiment (*cf.* Hevesy and Hahn (21)).

Later, Sacks changed his views concerning the relation between the P^{32} concentration of the inorganic phosphate and the labile organic phosphate. In the publication of 1942 (4) Sacks and Altschuler report (without any reference to the claims made in the paper of 1940) that the intracellular inorganic phosphate has an even lower P^{32} concentration than phosphocreatine and adenyl pyrophosphate. They found the plasma phosphate to have a P^{32} concentration 300 to 400 times that of the intracellular phosphate, the latter being calculated presumably according to the equations used in the present paper.

Sacks and Altschuler not only emphasized that after 30 minutes the phosphocreatine P and pyrophosphate in the resting muscle have the same isotope concentration as the inorganic phosphate in the muscle cell, but that the latter occasionally has a lower P^{32} concentration than the former. However, the few instances in which there was found a higher P^{32} concentration in phosphocreatine P than in inorganic phosphate show only a small difference (except in heart muscle) and, since the P^{32} concentration in the inorganic phosphate was based on calculations, it is doubtful whether such a small difference permits far reaching conclusions. Sacks and Altschuler draw the following conclusions concerning the phosphate turnover in resting and contracting muscle (after having stressed in the same paper that the P^{32} concentration of pyrophosphate and phosphocreatine is equal to or even higher than that of the inorganic phosphate (4)): "The present data indicate that if the phosphorylating glycolysis plays any part in the metabolism of muscle, it should be related to the resting metabolism, and not to activity metabolism." It should be clear, however, that the paper does not furnish any data whatsoever which would permit statements about the activity metabolism.

The inequalities in P^{32} concentration of phosphocreatine P and of pyrophosphate reported by Sacks, though they were not observed in any of the rabbit experiments in the present paper, are of interest, however. In Sacks' experiments the hexose monophosphate function shows the same variations in P^{32} concentration as are described in Table V in this paper. Sacks found high P^{32} values in this fraction 2 hours after the injection of radioactive phosphate, particularly when the latter contained large amounts of ordinary phosphate. It is reasonable to assume that the P^{32} concentration of the hexose monophosphate fraction is related to the amount of glycogen broken down. From the studies of Cori (17) we know that hexose monophosphate can be formed directly from glycogen and phosphate. The hexose monophosphate formed in this way can be expected to have a higher P^{32} concentration than that formed from phosphorylation of glucose by adenyl pyrophosphate. It is not excluded that the uptake of phosphate in the polysaccharide molecule (phosphorolysis) is a process of significance for the passage of phosphate from the outside in the muscle cell. Such a mechanism has recently been discussed by Hotchkiss (22) in connection with the uptake of phosphate in microorganisms. A phosphorolysis in the cell wall would explain the occurrence of higher P^{32} concentration in the hexose monophosphate fraction than in the intracellular inorganic phosphate (*cf.* Table V). Sacks' claim that inorganic phosphate enters into the intracellular inorganic phosphate only by hydrolysis of phosphocreatine or adenosine triphosphate seems to be without sufficient experimental basis.

The markedly lower P^{32} concentration in the terminal phosphate of adenosine triphosphate from frog muscle as compared with the second group is a phenomenon which is difficult to interpret. The results might be explained by assuming that in frog muscle the second phosphate group of adenosine triphosphate is rejuvenated mainly by inorganic phosphate (via the lactate cycle), while the terminal phosphate group is rejuvenated mainly by phosphocreatine. Another possibility is that both labile groups are equally rejuvenated from inorganic phosphate (via the lactate cycle) but that in frog muscle only the terminal group is being rejuvenated from phosphocreatine. Mincing the muscle gives rise to a large scale hydrolysis of phosphocreatine by way of the terminal group in adenosine triphosphate; the latter is then dephosphorylated by adenosine triphosphatase. This would give rise to a "washing out" of the original terminal phosphate (having the same P^{32} concentration as the second group) by phosphate of somewhat lower isotope concentration from the large phosphocreatine pool. A third possibility is that inorganic pyrophosphate is formed by phosphorylation of inorganic phosphate, and this fraction which would be included in the fraction of labile phosphate called group 2 would naturally have considerably higher P^{32} concentration than the organic labile phosphate. Cori and Ochoa (23) have recently discovered formation of inorganic pyrophosphate in liver, and Ferdmann and Feinschmidt (24) found that inorganic pyrophosphate is formed during muscular work and in the muscles of hibernating animals (25). Although the possibility of pyrophosphate formation ought to be kept in mind, it is held unlikely in the present case for the following reason. If inorganic pyrophosphate were formed, it would be found in the adenosine diphosphate fraction and the ratio of labile to total P as well as the N:P ratio should be greatly changed. This is not the case, however. From Paper I (13) it will be seen that labile to total P as well as the N:P ratio agrees with that of adenosine diphosphate.

The importance of investigating the phosphate fractions as early as possible after the injection of radioactive phosphate is well illustrated in the liver experiments. 5 minutes after the injection the pyrophosphate group in the adenosine triphosphate has attained 80 per cent of the P^{32} concentration of the intracellular inorganic phosphate.

Marshak's investigations (26) of the phosphorus turnover in nucleic acids furnish another example of the importance of using brief incubation time in case data on the rate of rejuvenation of phosphate compounds are wanted.

EXPERIMENTAL

Technique—The muscle tissue was deproteinized with 2.5 per cent $HgCl_2$ in 0.5 N HCl. The isolation of the adenine nucleotides and of hexose monophosphates has been described in Paper I (13).

Inorganic phosphate and phosphocreatine were always obtained from a small sample (1 to 5 gm.) of muscle tissue, which was fixed instantaneously in chilled trichloroacetic acid. Ammonium-magnesium mixture was added to the filtrate and inorganic phosphate was precipitated. The filtrate was acidified and ammonium molybdate was added in order to hydrolyze the phosphocreatine. The phosphocreatine phosphate was precipitated as ammonium magnesium phosphate and in some cases the supernatant fluid from the second precipitate was again made acid and pyrophosphate P hydrolyzed by 10 minutes boiling in acid. The liberated orthophosphate was precipitated as ammonium magnesium phosphate and the three precipitates were analyzed for isotopic phosphate.

In order to distinguish between organic esterified radioactive phosphate and impurities of strongly radioactive inorganic phosphate, the phosphoric ester to be investigated was subjected to a so called "washing" with ordinary inert orthophosphate. An amount of non-isotopic phosphate containing 2 to 3 times as much P as that of the phosphoric ester fraction was added and the phosphate then precipitated by addition of ammonium-magnesium mixture. If the P^{32} concentration decreased, it means that a part of the radioactivity of this fraction had been due to inorganic phosphates.

Determination of Radioactive Phosphate—Radioactive phosphate was determined in a Geiger-Müller counter. A 3 ml. sample was pipetted into a glass vessel which could be lifted up around a Bale immersion tube (27). The "background" was determined before and after each series and varied between 2 and 4 counts per minute. The amount of radioactive material was so chosen that net counts of between 10 and 100 per minute were obtained. The samples to be compared were either determined on the same day or the values obtained were corrected for the decay of P^{32}. The counts were always expressed as a percentage of the simultaneous P^{32} count in the inorganic phosphate of the muscle tissue.

Protocol 1—A rabbit weighing 4.5 kilos was anesthetized with 200 mg. of nembutal intravenously. A few minutes later 200 microcuries of radioactive sodium phosphate (0.6 mg. of P in 3 ml., pH 7) were administered intravenously. 15 to 20 minutes after the injection of radioactive phosphate, one cannula was tied in the aorta and another in the inferior vena cava; the femoral artery and vein of one leg were ligated and the leg removed and chilled in ice. The other leg was perfused with chilled mammalian Ringer's solution (phosphate-free) and the perfusion fluid from the leg was collected through the cannula in the vena cava. When 1 to 1.5 liters of fluid had passed through the leg, the perfusion was stopped and small samples of muscle tissue were obtained from the perfused as well as from the unperfused muscle. The main part of the muscles was then ground in a chilled meat grinder and deproteinized as described above.

Protocol 2—A rabbit weighing 3 kilos was injected intraperitoneally with radioactive sodium phosphate containing 500 microcuries. After 180 minutes the animal was anesthetized with nembutal. A blood sample was taken from the carotid artery and the animal was bled to death. The skeletal muscles were investigated according to the description above.

Protocol 3—A bullfrog weighing 250 gm. was anesthetized with urethane solution and then injected intravenously (vein in the abdominal wall) with radioactive phosphate (50 microcuries in 1 ml. containing 3.5 mg. of P). 15 minutes after the phosphate injection a blood sample was collected from the aorta, the circulation was stopped, a glass cannula placed in the aorta, and the hind legs were perfused for about 10 minutes with 1 to 1.5 liters of chilled (0°) frog Ringer's solution. The adductor muscles from both legs were frozen in dry ice, weighed, and extracted with chilled trichloroacetic acid for determination of inorganic phosphate. The main part of the muscles was extracted with mercuric chloride (5 per cent) in 0.5 N hydrochloric acid, and the adenosine triphosphate was isolated. The serum contained 27 mg. per cent of P (the greatly increased value being due to the injection of 3.5 mg. of P into the slowly circulating blood). The perfused muscle contained 16 mg. per cent of inorganic P.

Protocol 4—A well fed rabbit weighing 2.5 kilos was anesthetized with nembutal and then 500 microcuries of radioactive phosphate were injected into the jugular vein. A blood sample was collected from a mesenteric vein and 5 minutes after the injection of radioactive phosphate the liver was removed, chilled, and perfused with 1 liter of ice-cold Ringer's solution. 1 gm. of liver was extracted with trichloroacetic acid, the protein filtrate was neutralized with ammonia, and the inorganic phosphate was precipitated with magnesia mixture. The main part of the liver was minced and extracted with trichloroacetic acid and the protein-free filtrate then neutralized with barium hydroxide to pH 8.5. Adenyl pyrophosphate was isolated in the usual manner from the barium precipitate.

SUMMARY

The uptake of phosphate in muscle and the rate of rejuvenation of the labile phosphate groups have been studied by means of radioactive phosphate. It has been shown that in order to study the uptake and rejuvenation of phosphate in muscle it is necessary to remove the highly radioactive extracellular phosphate by perfusion.

The P^{32} concentration in the pyrophosphate group of adenosine triphosphate and in the phosphocreatine is more than half that of the intracellular inorganic phosphate 20 minutes after the injection of the P^{32}. The rate of penetration of phosphate in muscle was estimated to be approximately 1 γ of P per minute per gm. of muscle. The rate of rejuvenation of the labile phosphate groups was estimated to be 20 to 30 γ of P per minute per gm. of muscle.

The P^{32} concentration of the two labile phosphate groups in adenosine triphosphate has been determined separately by means of hexokinase which transfers only the terminal phosphate to glucose. In rabbit muscle the P^{32} concentration of the two labile phosphate groups was the same. However, in frog muscle at low temperature marked differences in the P^{32} concentration of the two labile phosphate groups in adenosine triphosphate occur. The terminal phosphate group attained usually the same P^{32} concentration as that of phosphocreatine, whereas the second group had a considerably higher P^{32} concentration. The reasons for this phenomenon have been discussed.

The rate of rejuvenation of labile phosphate in adenyl pyrophosphate in

liver is also very high. 5 minutes after injection of P^{32} the labile phosphate in adenyl pyrophosphate has attained a P^{32} concentration 80 per cent of that of the intracellular inorganic phosphate. The rate of rejuvenation was calculated to be about 15 γ of P per gm. of liver per minute.

The turnover of phosphate *in vivo* in various tissues was found to be much higher than that reported by a number of other authors. There are two main reasons for this disagreement. The first is that a number of investigators have failed to take into account the extracellular phosphate which must be separated from the intracellular phosphate. The P^{32} concentration of the organic phosphate has to be compared with the true intracellular inorganic phosphate.

The other reason is that until recently most investigators have been using by far too long an incubation time, from 1 to several hours, thus investigating the phosphate fractions a long time after they have reached the same P^{32} concentration as that of the intracellular inorganic phosphate. In the present paper the liver tissue was fixed 6 to 7 minutes after the injection of P^{32} in the animal and the labile phosphate of adenyl pyrophosphate had already attained a P^{32} concentration which was very near that of the intracellular inorganic phosphate.

The authors wish to express their gratitude to Dr. Sherwood Moore, Department of Radiology, and to Professor C. F. Cori, Department of Pharmacology and Biochemistry, Washington University School of Medicine, for their kind interest in this work.

Our thanks are also due to the members of the Department of Physics and the Cyclotron Laboratory, Washington University, for placing at our disposal amplifiers, Bale tubes, and radioactive phosphorus.

Finally, we want to thank Dr. S. P. Colowick, Washington University School of Medicine, and Dr. O. H. Lowry, the Public Health Research Institute of the City of New York, for their valuable criticism and suggestions while this paper was under preparation.

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THE ACONITE ALKALOIDS

XV. THE NATURE OF THE RING SYSTEM AND CHARACTER OF THE NITROGEN ATOM

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(Received for publication, April 6, 1944)

It has been fairly definitely established that aconine and delphonine, the alkamines of aconitine and delphinine, respectively, possess the formulations $C_{25}H_{41}O_9N$ and $C_{24}H_{39}O_7N$. The oxygen atoms are contained in hydroxyl and methoxyl groups and, since no unsaturation has been detected by hydrogenation studies, it has been concluded that these bases and others of the series, such as mesaconine, hypaconine, etc., are derived from a hexacyclic system which contains an alkylated nitrogen atom. The second group of alkaloids, such as atisine, staphisine, and kobusine, of much lower toxicity, which may predominate or accompany the above alkaloids in the original plant, has naturally been suspected to be structurally related to the first group. In the case of the second group of bases, the formulations have been determined only in certain instances with a fair degree of assurance. As a rule, they have been found from hydrogenation studies to be unsaturated, but in the case of heteratisine, which contains a lactone group, no unsaturation could be detected by this means. However, it is now well known that the failure to hydrogenate does not exclude the presence of resistant double bonds or other points of unsaturation. Since such information is so essential to the whole problem of the structure of these bases, we have sought to supplement the hydrogenation results by ultraviolet absorption spectra studies. This has yielded very interesting results.

The alkamines included in this study were aconine and delphonine, atisine, dihydroatisine, tetrahydroatisine, isoatisine, and heteratisine. In addition there were studied delphonine methochloride, its free quaternary base, the N-methyl-des base, and finally pyrodelphonine and α-oxodelphonine. With the exception of the delphonine derivatives, these substances have been described in previous publications (1). Since atisine and aconine were not crystalline, the bases were liberated from the crystalline hydrochlorides for the absorption study. Thus far, all attempts to prepare delphonine or its hydrochloride in crystalline form have been unsuccessful. However, a study of the saponification rates of delphinine has shown that the acetic and benzoic ester groups are very rapidly hydrolyzed when the alkaloid is heated with a slight excess of very dilute sodium hydroxide. It is probable that they are attached to either primary or secondary hydroxyl groups. The resulting *delphonine* could be distilled readily in a molecular still heated at 140°. The distillate appeared to have some of the characteristics of a crystalline substance and melted rather sharply at 76–78°. But all attempts to crystallize the base from a solvent or to obtain a crystalline salt were unsuccessful. The product so obtained was optically active and gave analytical data in agreement with the formulation $C_{24}H_{39}O_7N$. This preparation was used for the studies reported below.

Pyrodelphonine and *α-oxodelphonine*, also amorphous, were prepared in a similar manner by saponification of pyrodelphinine and α-oxodelphinine (2), respectively. *Delphonine methochloride* and its *hydroxide* were obtained in the usual way from the crude *methiodide*, which was formed by treatment of delphonine with excess methyl iodide. The *N-methyl-des base*, which was also not crystalline, was obtained by distilling the quaternary hydroxide in a molecular still.

The ultraviolet absorption spectrum curves obtained[1] with these substances were determined with a Beckman quartz spectrophotometer. These curves are presented in Figs. 1 to 7.

Aconine, delphonine, heteratisine, and also tetrahydroatisine in solution as bases all show a strong absorption within the range of 2200 to 2600 A, although it is of the uneventful type with no apparent maxima or minima. This is in the region of the spectrum where conjugated double bonds are known to absorb (3), but a maximum is usually to be expected with such conjugation. However, if a nitrogen is involved in some way with the double bonds, a somewhat different picture may be presented. This is shown by the absorption spectrum of pyrrole, which has the same type of absorption in the region we have investigated, but which also shows a maximum at shorter wave-lengths (4). Because of the limitations of our procedure and apparatus, we did not obtain data at such short wavelengths. But, even if maxima were present in this region in a more complicated molecule which contains many hydroxyl groups, they could be overshadowed by absorption from such groups. In our apparatus, N-methylpyrrole has given the type of absorption shown in Fig. 2. Since all of the oxygen atoms of these alkamines are present in hydroxyl or methoxyl groups, and the nitrogen atom is tertiary apparently with an alkyl group attached, it appears probable that the absorption must be due to a conjugated unsaturation of some kind.[3] However, all attempts to detect double bonds in these substances by hydrogenation have failed. Experience (3) has shown that a single isolated double bond is not sufficient to cause the amount and type of absorption in the region which has been found. If this behavior is correctly interpreted, it appears that two of the assumed six rings of these alkaloids (aconine and delphonine) may be accounted for by unsaturation of some type which is difficult to detect by hydrogenation, but which functions as a chromophorically active system. This would imply that the ring structure of the aconite alkaloids, at least in the form of free bases, could be of tetracyclic character.

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[1] In the preliminary stages of this work, a number of absorption spectra curves were kindly furnished us by Dr. G. I. Lavin, and determined with a Spekker spectrophotometer and a small Hilger quartz spectrograph. The curve of dihydroatisine given in Fig. 6 was one of these.

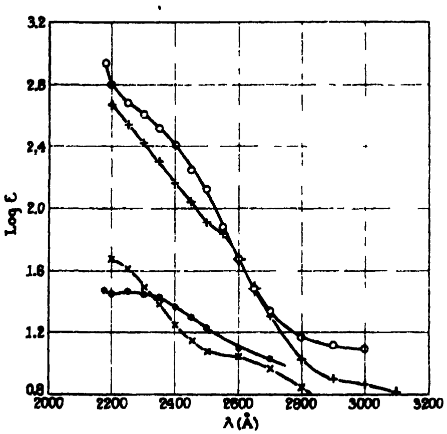

Fig. 1

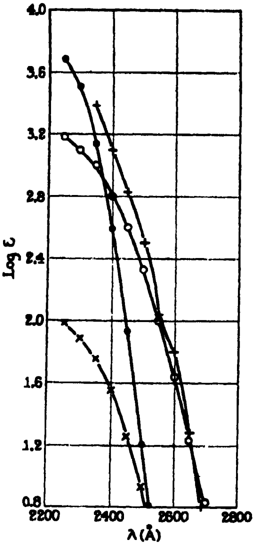

Fig. 2

Fig. 1. + = delphonine in dilute NaOH; ×, in dilute HCl. ○ = aconine in dilute NaOH; ●, in dilute HCl.

Fig. 2. ● = N-methylpyrrole in ethanol. + = tetrahydroatisine in ethanol. ○ = heteratisine in ethanol; ×, in ethanol containing HCl.

In a previous paper (6) of the series, the possibility of a relationship between the simpler aconite alkaloids and the diterpenes has been suggested. If this possibility should prove to be correct, then the aconite alkaloids could be built up from the three rings present in the diterpenes by addition of a fourth heterocyclic ring which contains the tertiary nitrogen. While such a view has many attractive possibilities from the standpoint of structure, it must be regarded, at the moment, as only a tentative, working hypothesis.

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[3] The recent experience of Klotz (5) with a cyclopropane ring conjugated to a double bond suggests that such an arrangement is a possibility which cannot be excluded in this problem.

The correct derivation and interpretation of the formulations of the aconite bases as a class now become of greater importance. On the basis of available data, it has been assumed heretofore that the alkamines of the more complicated aconitine alkaloids contain 19 carbon atoms (7) exclusive of those contained in methoxyl groups and the alkyl group attached to the nitrogen. However, in this respect a number of the simpler bases, *viz.* heteratisine (1), hetisine, and kobusine (8), appear to contain 20 carbon atoms, while others, such as atisine, napelline, and staphisine, appear to contain 21 carbon atoms unless the N-alkyl group should prove to be ethyl instead of the assumed methyl group. Formulations containing 20 carbon atoms would fit in with the postulated relationship to the diterpenes. Even if it should be confirmed that aconine, delphonine, and the like are derivatives of a nitrogen-containing hydrocarbon of 19 carbon atoms, this could be explained through loss of one of the diterpene methyl groups. The derived formulations of some of these alkaloids are still open to question, especially in the case of staphisine and napelline, and perhaps atisine. All must now be critically reexamined and the exact nature of the N-alkyl group assumed to be methyl must be determined. Such work is in progress in this laboratory.

In order to make the absorption spectrum studies complete, we have determined the absorption of the alkaloids in acid as well as in basic solution,[3] and striking changes have been noted. It was found that those alkamines, *viz.* aconine, delphonine, heteratisine, and tetrahydroatisine, which as free bases appeared to possess two conjugated double bonds or points of unsaturation, all showed as salts a marked shift in the position and intensity of the absorption. The hydrochlorides of these bases now absorbed in a manner which could be ascribed possibly to a single double bond as modified by the presence of the nitrogen and the hydroxyl groups present. Irrespective of such interpretation, an examination of Figs. 1 and 2 will show the striking shift in the absorption, due to the presence of excess acid. In the case of tetrahydroatisine, the absorption was so weakened that it could not be measured as the salt in excess hydrochloric acid. It therefore would seem that the double bonds or points of unsaturation in these bases are adjacent or connected in some way to the nitrogen atom. It has already been shown in the case of kobusine that double bonds are associated with its nitrogen atom (8). N-Methylpyrrole (Fig. 2) did not show an appreciable shift in acid solution.

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[3] It was found by experience that an appreciable excess of HCl above that required to form the salt had little effect on the absorption of the salt. Likewise, alkali in excess of that required to liberate the base had little effect on the absorption of the base. For convenience, therefore, the exact amount of excess acid or alkali is not recorded. Final concentrations of acid or alkali were never greater than 0.01 M.

In connection with these observations, it was of interest to determine the dissociation constants of several of these bases, since it is well known (9) that the proximity of a double bond to a basic nitrogen can reduce the strength of the base. The pH at 24° of solutions of delphonine partially neutralized with HCl was determined by use of the glass electrode.[4] In Fig. 3, the relationship of pH to the degree of neutralization is shown. At the half neutral point, the pH proved to be 10.02, which shows delphonine, in spite of its unsaturation, to be a surprisingly strong base and of the same order of strength as trimethyl- or triethylamine (10), which at the half neutral point have given pH values of 9.76 and 10.70, respectively. This might be explained by the assumption that in aqueous solution the alkaloid exists as an equilibrium mixture in which a small percentage is present with the nitrogen in a quaternary form.

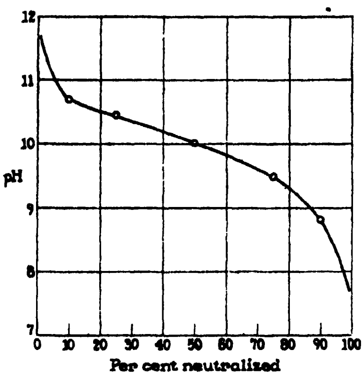

FIG. 3. Titration of delphonine

Adams and Mahan (11) have recently studied a series of tertiary vinyl cyclic amines and found them to be unexpectedly strong bases, of about the order we have now observed with delphonine. They explained the unexpected strength by the theory that the bases exist in solution as an equilibrium mixture, consisting of the tertiary unsaturated base and a quaternary rearrangement product in which the double bond has migrated to the nitrogen. This appears to be a logical explanation of the behavior which they encountered with their bases, and it would offer a good explanation for the behavior we have found with delphonine, as far as its basic strength is concerned.

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[4] We are indebted to Dr. Alexandre Rothen, of The Rockefeller Institute for Medical Research, for making these measurements for us.

Whether or not such a theory would adequately explain the change of absorption spectrum with pH, which has been observed with our bases, is not at present entirely clear. The marked reduction of the ultraviolet absorption in acid solution might be due to the migration of one of the double bonds to an unconjugated position, to the disappearance of a point of unsaturation through ring formation, or to some other unexplained rearrangement. Marked shift of absorption spectrum with change of pH is not uncommon with unsaturated nitrogen heterocycles, in which several resonating forms are possible, and has been observed with pyrimidines (12), aniline (13), vitamin B_6 (14), and others, although these systems are more or less aromatic in character.

The dissociation constant of aconine was found to be slightly less striking ($pH = 9.52$ at the half neutral point), but still much higher than would be expected if double bonds are associated with the nitrogen. The shift of the absorption spectrum with change of pH was entirely analogous (Fig. 1) to that found for delphonine.

The behavior of α-oxodelphonine has a definite bearing on the observations thus far described. α-Oxodelphonine differs from delphonine (at least in part) by having 2 hydrogens presumably on a carbon atom adjacent to the nitrogen replaced by an oxygen, and is thus a cyclic amide, since it exhibits no marked basic properties. It shows an absorption spectrum which is similar to that of delphonine in alkaline solution (Fig. 4). Although the absorption is somewhat more intense in the shorter wave-lengths, it is somewhat less in the longer wave-lengths. It is suggested by the type of curve shown that the CO group in the amide linkage is not conjugated to the two other conjugated points of unsaturation. No appreciable shift was observed when the solution was made acid with HCl.

The effect on the ultraviolet absorption of the methylation of the nitrogen of delphonine has also been studied. Although delphonine methiodide could not be crystallized, it was converted to the quaternary base by removal of iodine with excess silver oxide. After removal of solvent, the base was directly used for the absorption spectrum study shown in Fig. 5. The quantitative aspects of these curves are somewhat less reliable because of manipulative difficulties with the substance, but the general picture appears to be clear cut. There was a much smaller shift in the absorption spectrum over most of the range when the solution was made acid, but the result seemed to be somewhat clouded by another type of equilibrium which caused absorption in the longer wave-lengths. A pale yellow color was present in alkaline solution, which disappeared on acidification and reappeared on addition of alkali. The solutions appeared to be comparatively stable.

When the quaternary base was distilled in a molecular still, a distillate was obtained in good yield which, like delphonine, could not be crystallized,

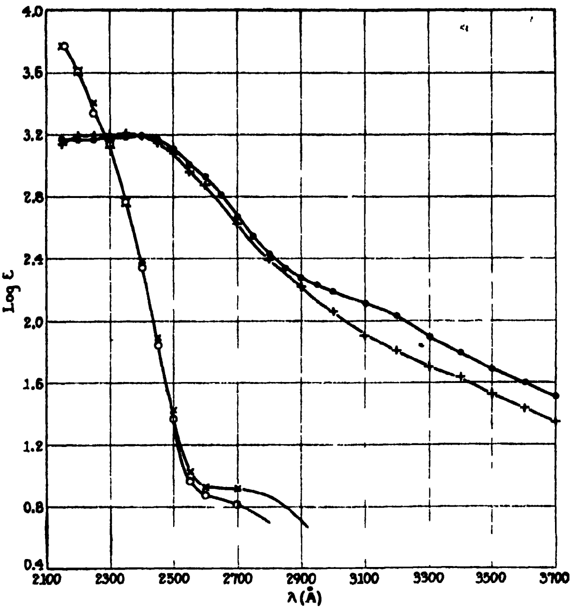


FIG. 4. ○ = oxodelphonine in H_2O; ×, in dilute HCl. ● = N-methyl-des-delphonine in H_2O; +, in dilute HCl.

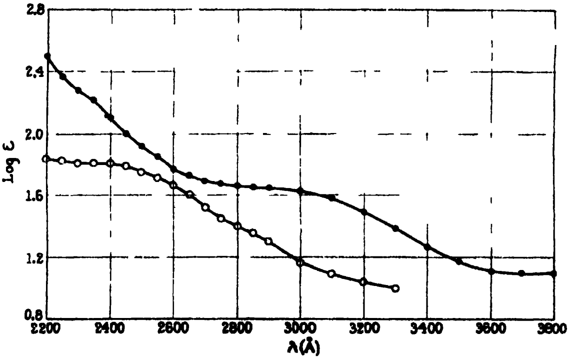


FIG. 5. ○ = delphonine methochloride in dilute HCl; ●, in dilute NaOH

but which gave good analytical data for the N-methyl-des base. Such a base should normally possess a double bond in addition to those present in delphonine. The absorption spectrum observed (Fig. 4), as well as the analytical data, strongly indicates the presence of such an additional double bond, since the absorption was shifted greatly toward the longer wave-lengths. The striking observation was now made that only an inappreciable shift of the absorption appeared on acidification.

All attempts to carry the Hoffmann degradation beyond this point, in order to eliminate the nitrogen, have been unsuccessful. The nitrogen must, therefore, be either so closely associated with double bonds or so highly hindered as to interfere with such degradation.

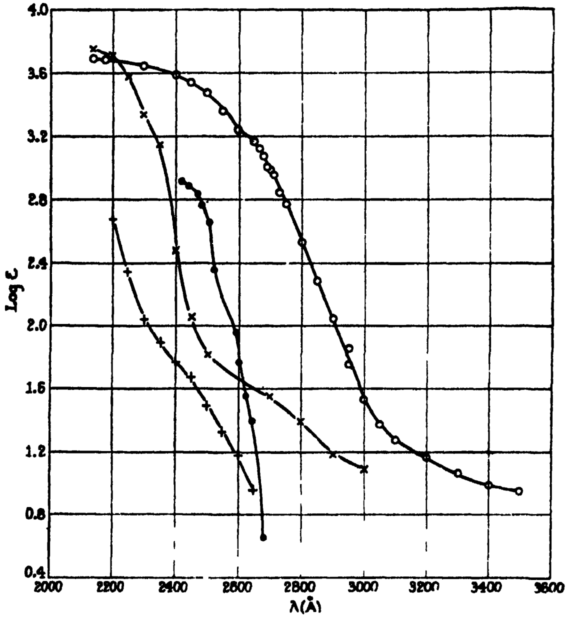


FIG. 6. ○ = pyrodelphonine in H_2O; ×, in dilute HCl. ● = dihydroatisine in ethanol; +, in ethanol with HCl added.

Pyrodelphonine, obtained by saponification of pyrodelphinine, differs from delphonine empirically by a molecule of water. The absorption spectrum of this base (Fig. 6) suggests that the difference in structure consists in the presence in the pyro compound of a new double bond which is conjugated in some way with those already present in delphonine since, as in the case of N-methyl-des-delphonine, the absorption is displaced greatly toward the longer wave-lengths. However, this absorption does not coincide with that of the latter compound, and a large shift is observed on acidification. The arrangement of the double bonds, therefore, would appear to be different than in the case of the N-methyl-des base. The absorption in acid solution is now quite similar to that observed for delphonine in alkaline solution, an observation not unexpected if acidification relieves one of the double bonds of the ability to contribute to the absorption at the wave-lengths under consideration. In the latter case, two conjugated double bonds could still remain. Pyrodelphonine was found to be a considerably weaker base (pH = 8.21 at the half neutral point) than delphonine.

The absorption curves of atisine, isoatisine, and dihydroatisine (Figs. 6 and 7) all resemble each other in general form, although there appears to be no maximum or minimum. All three contain double bonds which can be recognized by hydrogenation. All three show a certain shift upon acidification. The shift of the curve for dihydroatisine is in the same direction noted in the case of delphonine, but the hydrochloride still strongly absorbs. Atisine and isoatisine show stronger absorption as the salt in acid solution than as the free base in alkaline solution.

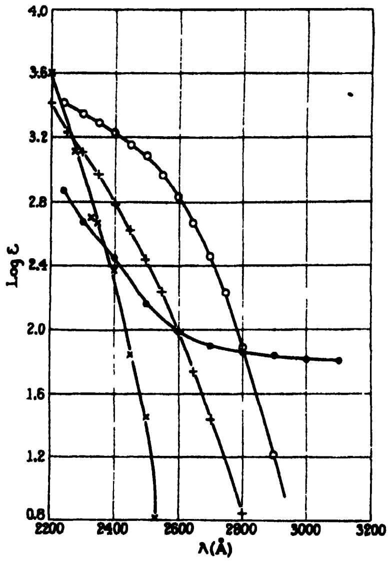
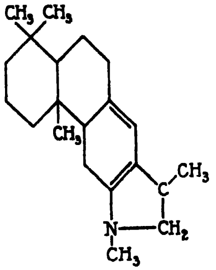


FIG. 7. ○ = atisine hydrochloride in ethanol; ●, in ethanol with excess NaOH added. + = isoatisine hydrochloride in ethanol; ×, in ethanol with excess NaOH added.

Heteratisine gives a similar type of absorption (Fig. 2). It also shows a similar shift upon acidification.

The studies described above are not in themselves sufficient to derive a special type of structure, but they appear to limit the possibilities which may be considered in the derivation of the structures of these alkaloids, especially if the assumption of a relationship to the diterpenes should prove to be correct. Although such a relationship is indicated only by the evidence obtained with the atisine type of base, it may be inferred also in the case of the aconine type of bases. The resemblance in structure between the two types already probable, since they occur together in the same plants, appears now strengthened by the indication that the configuration involving the nitrogen must be similar in order to show the unique absorption spectrum behavior given above.

The data which have accumulated might serve as a basis for further speculation regarding the structure of this little understood group of alkaloids. If the relationship to the diterpenes (6) should prove to be correct, only certain structures would be possible within the limitations imposed by the empirical formulas and the data given above. One of these possibilities is presented, merely as an example, in Formula I. The positions of the nitrogen and of the heterocyclic ring have been arbitrarily chosen. The hypothesis that they involve the isopropyl side chain would afford sufficient flexibility in structure to explain the data presented in this paper.



Formula I

EXPERIMENTAL

Delphonine—2 gm. of crystalline delphinine were dissolved in 20 cc. of hot ethanol, and 10 cc. of 1.056 N NaOH were added. The solution was heated for 5 minutes on the steam bath. The ethanol was then quickly removed under reduced pressure, and the alkamine was extracted from the alkaline solution with ether. The concentrated extract was distilled in a molecular still. Approximately 1 gm. of material distilled in about an hour at an oil bath temperature of 140°, and with the pressure varying from 0.001 to 0.0001 mm. The distillate formed a brittle, possibly semicrystalline resin, with a rather sharp melting point of 76–78°. It could not be induced to crystallize from any solvent.

$[\alpha]_D^{24} = +37.5°$ ($c = 3.2$ in ethyl alcohol)

$C_{24}H_{39}O_7N$. Calculated. C 63.53, H 8.67, OCH_3 27.35, $(N)CH_3$ 3.31
Found. " 63.44, " 8.37, " 27.60, " 3.34

Pyrodelphonine—2 gm. of pyrodelphinine (2) were hydrolyzed as given above for delphinine. Although the substance was rather sparingly soluble in the hot ethanol, solution rapidly occurred on addition of the alkali. 0.73 gm. of material distilled in the molecular still in a little over an hour under conditions similar to those of the previous case. The distillate formed a resin which could not be crystallized from any solvent.

$C_{24}H_{37}O_6N$. Calculated. C 66.16, H 8.57, OCH_3 28.49
Found. " 66.06, " 8.52, " 27.95

α-Oxodelphonine—α-Oxodelphinine (2) was hydrolyzed in the same manner. A slightly higher temperature (170–180°) than in the case of the above substances was required for the distillation of the resulting product. The distillate had much the same appearance, and likewise could not be crystallized from any solvent.

$C_{24}H_{37}O_8N$. Calculated. C 61.63, H 7.98, OCH_3 26.53, $(N)CH_3$ 3.21
Found. " 61.36, " 7.89, " 26.31, " 0.85

The very low $(N)CH_3$ determination is in agreement with the former experience with the oxodelphinines (2).

N-Methyl-Des-Delphonine—A solution of delphonine in excess methyl iodide was heated in a sealed tube at 100° for 3 hours. A resin of the methiodide separated. After removal of excess methyl iodide, the resin was dissolved in methanol. The halogen was removed from the solution with excess silver oxide. The filtrate was concentrated and placed in a molecular still. A good yield of material distilled at a bath temperature of 180–200° under a pressure varying from approximately 0.01 mm. to 0.0001 mm. during a little over an hour. The distillate formed a resin which could not be crystallized.

$C_{25}H_{41}O_7N$. Calculated. C 64.20, H 8.86, OCH_3 26.53, $(N)CH_3$ 6.42
Found. " 64.25, " 8.62, " 26.04, " 4.40

All analyses were performed by Mr. D. Rigakos.

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LETTERS TO THE EDITORS

THE NON-APPEARANCE OF SALICYLATES IN THE URINE AFTER THE ADMINISTRATION OF DICUMAROL

Sirs:

The production of a hypoprothrombinemic state by dicumarol [3,3′-methylenebis(4-hydroxycoumarin)],[1] the *in vitro* degradation of dicumarol to 2 equivalents of salicylic acid,[1] and the demonstration of a hypoprothrombinemic state following administration of large doses of salicylates[2] have led to the suggestion that the hypoprothrombinemia following dicumarol occurs from the formation of salicylates. The occurrence of salicylates in the urine after administration of dicumarol has not been reported. Since the major portion of administered salicylate is excreted in the urine, the urine of rats given dicumarol was analyzed for total salicylates. The method, to be described later, is similar to that of Brodie, Udenfriend, and Coburn,[3] but involves the ether extraction of a sulfuric acid hydrolysate and the colorimetric estimation of the salicylic acid in the residue of the ether extract with ferric chloride. Dicumarol does not interfere in the analytical determination.

Rats maintained on the same stock diet were divided into three groups: those of one group received daily for 3 days 25 mg. of synthetic dicumarol[4] suspended in 1 cc. of 10 per cent gum acacia; another group, 25 mg. of sodium salicylate dissolved in 1 cc. of 10 per cent gum acacia; and a third group, as controls, 1 cc. of 10 per cent gum acacia only. The urines were pooled each day from each group, acidified, and kept at 6° until analyzed.

The rats given sodium salicylate excreted 21.0 mg. of salicylate (as sodium salicylate) per day per rat. Those given dicumarol excreted none.

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[1] Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 513 (1941).

[2] Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, **147**, 463 (1943).

[3] Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol. and Exp. Therap.*, **80**, 114 (1944).

[4] Supplied through the courtesy of Werner Bergmann, Sterling Chemistry Laboratory, Yale University.

This finding, as to dicumarol, is substantially in agreement with the results of Sullivan and Huebner.[5]

Blood was drawn on the 4th day, 24 hours after the last administration and the prothrombin time of 12.5 per cent plasma determined.[6] That for the rats used as controls averaged 32.1 seconds, for the rats given salicylate, 27.2 seconds, and for the rats given dicumarol, no clotting occurred within 5 minutes. The rats given dicumarol died on the 5th day.

The recent work of Kabat, Stohlman, and Smith[7] has shown that indandione and its derivatives are similar in hypoprothrombinemic action to dicumarol and that 2-pivalyl-1,3-indandione, in particular, is practically as effective as dicumarol, it would be highly unlikely that these substances would yield salicylic acid as a metabolite. If a substance exerts an effect through a metabolite, it may be assumed that the metabolite is at least as potent in causing the same result, dicumarol is, however, 25 to 100 times as effective as the salicylates in producing hypoprothrombinemia. It is also well recognized that vitamin K in a small dose will protect the rat against the hypoprothrombinemia from a single large dose of salicylate, while a massive dose of the vitamin is required to counteract the effects of a single small dose of dicumarol.

The present results, taken together with the arguments outlined above, strongly indicate that dicumarol does not cause hypoprothrombinemia through the intermediation of salicylates

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David Lester

Received for publication, March 18, 1944

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[5] Sullivan, W. R., and Huebner, C. F., private communication from Karl P. Link.

[6] Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1 (1941). Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **142**, 941 (1942).

[7] Kabat, H., Stohlman, E. F., and Smith, M. I., *J. Pharmacol. and Exp. Therap.*, **80**, 160 (1944).

MICROBIOLOGICAL METHOD FOR THE QUANTITATIVE DETERMINATION OF SMALL QUANTITIES OF POTASSIUM

Sirs:

The essential nature of the potassium ion for plant life has been known for some time. Recently it was found that the growth of yeasts, and the growth, acid production, and pH change by *Lactobacillus casei* No. 7469, respond quantitatively to increasing concentrations of potassium. It was also found that sodium and the ammonium ion do not replace potassium and do not interfere with the quantitative response to potassium by these organisms. Because of these facts and also because the difficulty of determining potassium in the presence of certain other ions is conceded, it seemed profitable to devise a microbiological method for the quantitative determination of small quantities of potassium.

The medium used contains casein hydrolysate, glucose, phosphate, adenine, guanine, xanthine, uracil, sodium acetate, asparagine, tryptophane, cystine, methionine, mineral salts, and vitamins, but no added potassium. It is a modification of the medium employed by the author.[1] The assay procedure was similar to that of microbiological methods for the assay of vitamins and amino acids. Especially purified KCl was used in the medium and a standard curve was constructed from the results of acid production or pH change at levels of 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 γ of K^+ per ml. of medium.

The salts noted in the table were dried and solutions of each were made to the designated concentrations. The assayer was not informed as to the nature of the salts and knew them only by code letters.[2] He was told only

Results of Microbiological Assay for Potassium

Salt	Quantity of potassium		
	Present	Found	Difference
	γ per ml.	*γ per ml.*	*per cent*
KNO_3	167.7	169	+0.78
KCl	145.6	140	−3.85
"	161.0	166	+3.10
KH_2PO_4	149.2	154	+3.22
$NaKC_4H_4O_6$	94.9	92	−3.06

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[1] Rogosa, M., *J. Bact.*, **47**, 159 (1944).

[2] The author is indebted to G. A. Ramsdell for making the solutions and coding the samples.

that any one of the solutions might contain between 100 and 200 γ of K^+ per ml.

It should not be concluded at this time that this method will uniformly be as precise as the table indicates or that the method can be applied routinely to biological materials. Experiments extending the method to biological materials are in progress and possible non-specific stimulation is being investigated. The general principle of the method has been valid thus far and it is hoped that further investigation will permit the method to be applied generally.

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Received for publication, April 25, 1944

THE RELATIONSHIP OF ADENOSINE POLYPHOSPHATES TO FATTY ACID OXIDATION IN HOMOGENIZED LIVER PREPARATIONS

Sirs:

The failure or only partial success of some investigators[1] to obtain reproducible oxidation of the higher fatty acids by broken cell preparations of liver can be explained by assuming that adenosine triphosphate (or diphosphate) is required to initiate fatty acid oxidation. The basis for this assumption is implicit in their data and certain other considerations.

It was found possible to demonstrate, in quantitatively reproducible experiments, considerable extra oxygen uptake due to added normal saturated fatty acids having from 4 to 18 carbon atoms by rat liver homogenates supplemented with cytochrome. This occurred only when ATP, ADP, the corresponding inosine nucleotides, or compounds capable of phosphorylating adenylic acid were added. Adenylic acid alone was inactive. This activation could also be demonstrated by aerobic phosphorylation of adenylic acid by oxidation of pyruvate or fumarate in liver homogenates fortified with Mg^{++}, K^{+}, diphosphopyridine nucleotide, and cytochrome.

The rate of oxidation was found to depend on the adenosine polyphosphate concentration; optimum results were obtained at 0.0025 M, a concentration comparable to that existing in quick frozen intact liver.

The activation of octanoic acid oxidation by ATP is shown in a typical experiment: 0.20 ml. of H_2O or sodium octanoate solution was tipped into 0.25 ml. of rat liver homogenate (chilled liver homogenized with 2 volumes of Krebs-Ringer phosphate buffer, pH 8.0, without Ca^{++}), 0.25 ml. of ATP (or water), 0.10 ml. of cytochrome solution, and 0.20 ml. of 0.13 M phosphate buffer, pH 8.0. Final concentrations, 4×10^{-5} M cytochrome, 0.0025 M ATP, 0.0025 M octanoate. Time 30 minutes; temperature 25°. Gas phase, air. Octanoate was determined by distillation.

System	Substrate	O_2 uptake	Octanoate removed
		micromoles	*micromoles*
1. Complete + ATP........	−	2.4	
2. " + "	+	7.0	1.72
3. " − "	−	2.4	
4. " − "	+	1.6	−0.13

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[1] Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 27, 1753 (1933). Leloir, L. F., Muñoz., J. M., *Biochem. J.*, 33, 734 (1939). Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, 147, 355 (1943).

The mechanism of the activation is being investigated and the data obtained suggest the intermediate formation of acyl phosphates. Preliminary experiments with synthetic acyl phosphates having from 8 to 16 carbon atoms indicate that they do not require ATP for oxidation in the above system. After hydrolytic destruction by acid, these synthetic preparations do require ATP activation. Final crucial experiments to test this hypothesis await complete purification of the acyl phosphates.

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Received for publication, May 8, 1944

THE UTILIZATION OF ACETIC ACID FOR FATTY ACID SYNTHESIS

Sirs:

No information is available as to the intermediates in the biological synthesis of fatty acids from carbohydrates. In earlier experiments[1] with dueterio acetate the isotope concentrations found in the fatty acids were not significant. We have now prepared a sodium acetate containing 19.6 atom per cent excess C^{13} in the carboxyl group and a high concentration (77 atom per cent excess) deuterium in the methyl group. This acetate was fed to mice at a level of 1.6 mM per 100 gm. of body weight for 8 days. Fatty acids were then isolated from the pooled livers and skinned carcasses. A sample of respiratory CO_2 was obtained 15 minutes before the end of the experimental period. Glycogen (11.3 mg.) was isolated from the livers.

From the total fatty acids of the liver (238 mg.), 48 mg. of saturated fatty acids were isolated. After their analysis for C^{13} and D, the remaining 44 mg. were decarboxylated[2] in order to determine the concentration of C^{13} in the carboxyl group. The fatty acids of the skinned carcasses (2.3 gm.) were separated in a similar manner. The isotope concentration of the various fractions are given in the table.

Sodium Acetate Fed, 9.8 Atom Per cent Excess C^{13}, 77 Atom Per Cent Excess D

	Atom per cent excess			
	Carcass		Liver	
	C^{13}	D	C^{13}	D
Total fatty acids	0.081	0.13	0.103	0.32
Saturated fatty acids	0.101	0.24	0.160	0.42
Carboxyl carbon of saturated fatty acids	0.179		0.290	
Glycogen			0.038	

The dueterium concentration of the body fluids at the end of the experiment was 0.09 atom per cent excess. The respiratory CO_2 during the last 15 minutes contained 0.066 atom per cent excess C^{13}.

A similar experiment was carried out with growing rats. They were fed for 3 days acetate containing 9.8 atom per cent excess C^{13} at a level of 1.6 mM per 100 gm. of body weight. The total fatty acids contained 0.040 atom per cent excess C^{13} and the carboxyl carbon of the total acids contained 0.090.

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[1] Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **145**, 625 (1942).

[2] Easterfield, T. H., and Taylor, C. M., *J. Chem. Soc.*, **99**, 2298 (1911).

In connection with these findings, the work of Sonderhoff and Thomas with yeast[3] and the short time experiments of Buchanan, Hastings, and Nesbett[4] with rats are of interest. The results here reported furnish positive evidence for the utilization of the carbon of the carboxyl group of acetic acid for fatty acid synthesis.

On complete combustion, the carbon of the diet of the mice (0.5 atom of carbon per day per 100 gm. of body weight) should yield CO_2 with an average C^{13} concentration of about 0.07 atom per cent excess. As the concentrations found in the fatty acids are in all cases higher than this value, the results cannot be due to CO_2 assimilation. Moreover, as after the feeding of the deuterio acetate for 8 days the fat had a deuterium concentration of 1.5 to 4.5 times that of the body fluids, utilization of the methyl, as well as the carboxyl, groups of acetate must have occurred. The fact that the C^{13} concentration of the carboxyl carbon atom is approximately twice that of the total molecule suggests that the fatty acids are synthesized by successive condensations of C_2 units.

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Received for publication, May 19, 1944

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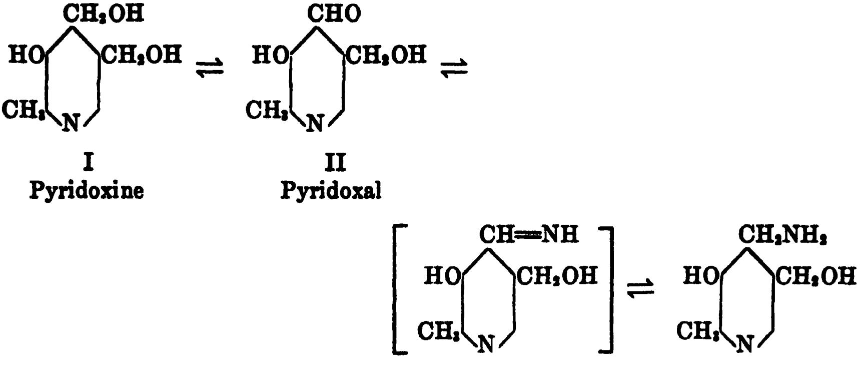
[3] Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).

[4] Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413 (1943).

THE VITAMIN ACTIVITIES OF "PYRIDOXAL" AND "PYRIDOXAMINE"

Sirs:

It was earlier demonstrated[1] that one or more substances occur naturally which have far greater activity in promoting growth of lactic acid bacteria in a pyridoxine-free medium than does pyridoxine itself. Our investigations revealed that mixtures with heightened growth-promoting activity for lactic acid bacteria were formed from pyridoxine by (*a*) procedures causing possible amination of pyridoxine and (*b*) those causing partial oxidation.[2] Indirect evidence showed the active compound produced by amination to be an amine; that by oxidation, an aldehyde. Adoption of a hypothesis providing for biological interconversion of the active compounds (see below) reduced the possible structures to three each for the amine and the aldehyde. Synthesis of four selected compounds of definite structure should test the hypothesis decisively, and furnish the two active products.



I
Pyridoxine

II
Pyridoxal

III

IV
Pyridoxamine

At this stage in the investigation, cooperation of the research staff of Merck and Company, Inc., was enlisted. Biological test of the compounds prepared by them[3] revealed 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine (II) and 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine (IV) to be the active aldehyde and amine, respectively (see the table). When pyridoxamine or pyridoxal is used as a standard of comparison with *S. lactis*, values for the "B_6" content of natural materials are obtained similar to those indicated by yeast assay, instead of the absurdly high values obtained against a pyridoxine standard.[1] Considerable evidence indicates that these compounds or their higher combinations are responsible for the "pseudopyridoxine" activity of natural materials.

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[1] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143**, 519 (1942).

[2] Carpenter and Strong (*Arch. Biochem.*, **3**, 375 (1944)) have also shown that mild oxidation of pyridoxine yields a substance with increased activity for *L. casei*.

[3] Harris, S. A., Heyl, D., and Folkers, K., *J. Biol. Chem.*, **154**, 315 (1944).

Organism*	Comparative activity		
	Pyridoxine hydrochloride	Pyridoxal	Pyridoxamine
Streptococcus lactis R	1.0	5000–8000	6000–9000
Lactobacillus casei.	1.0	1000–1500	3–10
Saccharomyces carlsbergensis	1.0	0.9–1.4	0.8–1.3

* Assays with *S. lactis* and *L. casei* were performed essentially as described previously[1] except that samples were added aseptically after sterilization of the medium.[4] Assays with yeast were performed by a slight modification of the method of Atkin *et al.*[5]

A plausible hypothesis is that transformation into pyridoxal, pyridoxamine, or substances derived from them is required for fulfilment of the catalytic function of pyridoxine in all organisms. Organisms such as yeast, for which pyridoxine has full activity, effect this transformation without difficulty; most lactic acid bacteria do not. This hypothesis suggests possible catalytic rôles for pyridoxine. Reversible transformation of I into II or IV into III might permit function in hydrogen transport. Interconversion of II and IV may occur by transamination reactions[6] without involving the intermediate III; a rôle in biological transaminations is thus a possibility.

The University of Texas, Biochemical Institute,
and the Clayton Foundation for Research
Austin

Esmond E. Snell

Received for publication, May 8, 1944

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[4] Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, **51**, 356 (1942).

[5] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

[6] Braunstein, A. E., *Enzymologia*, **7**, 25 (1939). Herbst, M., and Rittenberg, D., *J. Org. Chem.*, **8**, 380 (1943).

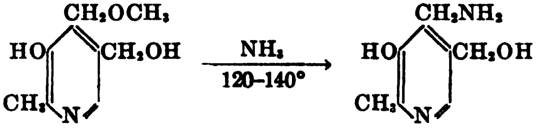
THE STRUCTURE AND SYNTHESIS OF PYRIDOXAMINE AND PYRIDOXAL

Sirs:

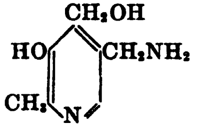
Data submitted to us by Dr. E. E. Snell showed that various procedures involving amination or oxidation of pyridoxine resulted in substances which had much greater activity than pyridoxine itself toward certain lactic acid bacteria. His studies indicated that the active product obtained by amination was an amine and the one from oxidation an aldehyde, and that there were only three possible structures for each of these compounds.[1]

As a result of this collaboration with Dr. Snell, we have succeeded in synthesizing both an active amine and an active aldehyde, and have proved that they are 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine and 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine respectively. The former has been named pyridoxamine and the latter pyridoxal.[1]

Amination of pyridoxine diacetate[2] yielded an active amine, m.p. 193–193.5°. As previous investigations[2,3] had shown that vitamin B_6 and its derivatives are very reactive toward substitution on the 4-methylene group, we anticipated the formation of the 4-aminomethyl derivative II. The best yields of this compound were obtained by the amination of 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine,[2] I. The loss of the methoxy group also indicated substitution on the 4-methylene position.



I II



III

The isomeric 2-methyl-3-hydroxy-4-hydroxymethyl-5-aminomethylpyridine III (m.p. 176–178°) was synthesized by converting the 4-methoxymethyl compound I by means of thionyl chloride into the 5-chloromethyl derivative, treating this with ammonia, and subjecting the resulting 4-methoxymethyl-5-aminomethyl compound to hydrolysis.

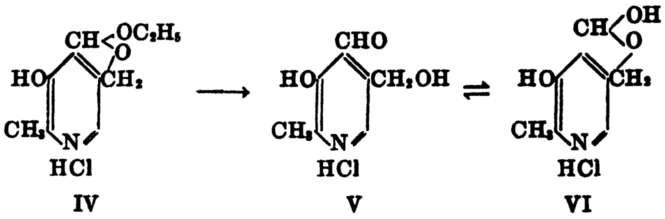
The oxidation of pyridoxine with potassium permanganate yielded an aldehyde which was isolated as its oxime; m.p. 225–226°. On decomposition of the oxime with nitrous acid and subsequent treatment with ethanol and hydrochloric acid, the cyclic acetal IV was obtained. This compound

[1] Snell, E. E., *J. Biol. Chem.*, **154**, 313 (1944).

[2] Harris, S. A., *J. Am. Chem. Soc.*, **62**, 3203 (1940).

[3] Harris, S. A., *J. Am. Chem. Soc.*, **63**, 3363 (1941).

was easily hydrolyzed to the aldehyde, which may have either of the structures V or VI.



Proof that the formyl group was in the 4 position was obtained by catalytic hydrogenation of the oxime to the amine, II.

Microbiological assays of these compounds by Dr. Snell demonstrated the 5-aminomethyl compound III to be without significant activity. 2-Methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine II was shown to be the active amination product, pyridoxamine, and 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine V the active oxidation product pyridoxal.[1]

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Received for publication, May 8, 1944

FURTHER OBSERVATIONS ON PHYSIOLOGICAL ANTIOXIDANTS

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(Received for publication, February 25, 1944)

The study of antioxidants has only recently been applied to physiological reactions as they occur *in vivo*. α-Tocopherol, the most extensively investigated of these compounds, has been demonstrated to protect carotene in the intestine from the destructive action of oxidizing fat (1–3) and evidence has been presented that this vitamin might act under special conditions as a regulator of tissue oxidation (4). Soon after their discovery of natural antioxidants, the inhibitols, Olcott and Mattill found that the antioxygenic activity of these compounds for vegetable fats can be reinforced by additional acidic antioxidants (5). An example, possibly of physiological significance, was the enhancement of the antioxygenic activity of α-tocopherol by ascorbic acid (6). In the complex system of the animal body it seems more probable that inhibition of fat oxidation would be the result of the synergistic action of several factors rather than of a single entity. Such a synergism of antioxidants appears to play a rôle in the antioxygenic reactions involved in the production of malignant hepatoma in rats by feeding the dye, N,N-dimethylaminoazobenzene, commonly called butter yellow.

Antioxidant Activity of Butter Yellow

In a series of nutritional experiments on the relationship of diet to the production of cancer by butter yellow, it was found that malignant hepatoma would occur in rats upon the ingestion of butter yellow in a synthetic diet, provided the fat intake is kept low, or with a high fat ratio in the diet, provided the fat constituents were Crisco or butter fat with a relatively low percentage of unsaturated fatty acid (7). If lard was used, cancer was prevented but the protective effect of lard could be overcome by the inclusion of rice (especially unpolished) in the diet. *In vitro* experiments demonstrated that linoleic acid, the chief unsaturated fatty acid of lard, when it became rancid destroyed butter yellow, presumably as a result of coupled oxidation. Brown rice (and to a smaller extent polished rice) prevented the oxidation of linoleic acid and consequently preserved the butter yellow. In view of these results it was concluded that Crisco, butter fat, and rice, which might have a procarcinogenic effect, when fed with butter yellow, do so by stabilization of the carcinogenic dye either by reducing the content of unsaturated fatty acids in the diet or through a direct antioxygenic effect. The stabilization or its reverse, the destruction of the dye, takes place either in the food mixture itself or during the passage of food through the gastrointestinal tract. This second possibility is especially important, because it points to reactions and mechanisms of purely physiological nature. If rancidity of the food mixture alone would determine the anticarcinogenic effect of butter yellow, no great importance could be attached to its rôle in the production of cancer by butter yellow. This fact should be emphasized especially in view of possible misunderstanding (8) and of the finding that in the use of food mixtures containing large amounts of lard the anticarcinogenic effect was fully discernible in spite of complete absence of rancidity.

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In previous experiments (7) results were obtained which indicated that butter yellow itself possessed antioxidant activity. A diet low in casein, containing linoleic acid and butter yellow, proved to be toxic to rats although in no cases were hepatomata found. The rats fed the linoleic acid ration lost weight rapidly, became infested with pediculi, and developed marked progressive anemia usually accompanied by leucopenia. This toxicity was believed to be caused by the oxidation products of linoleic acid, a belief shared by Burr and Barnes (9) who recently reported similar anemia in rats fed rancid lard. Removal of the butter yellow from the diet resulted in an increase in the severity of symptoms. This observation might be explained by the assumption that butter yellow possesses antioxygenic activity. Rusch and his coworkers, studying the relationship of chemical nature to biological response in a number of carcinogens, found that butter yellow did not retard the autoxidation of cod liver oil or corn oil, but it inhibited the oxidation of benzaldehyde (10) and that of phospholipids in the presence of ascorbic acid as catalyst (11). In a recent study of natural antioxidants in sources of the vitamin B complex, activity was measured by the decrease in the iodine number and by the time required for the linoleic acid after it became rancid to decolorize a definite amount of butter yellow (12). Butter yellow was part of the system to be used for the assay of added antioxidants. The possibility that butter yellow itself exerts antioxidant activity and the physiological significance of such action warranted further investigation.

Experimental Methods and Results

The methods employed in this study have been described in the previous paper (12). 8.4 gm. of corn-starch were mixed with 1.6 gm. of linoleic acid.[1] Butter yellow when added was dissolved in linoleic acid. This system was allowed to undergo oxidation at 30°, the O_2 uptake being followed by the decrease in the iodine number. The destruction of butter yellow was measured colorimetrically in chloroform extracts.

The corn-starch-linoleic acid system, with or without butter yellow, proved to be, as in the previous studies (12), a valuable and simple approach to the quantitative measurement of antioxidant activity. The results could easily be repeated and, although the absolute figures for iodine number or for the concentration of butter yellow varied slightly in duplicate experiments, the results were sufficiently quantitative to warrant unequivocal conclusions. Negative controls, *i.e.* corn-starch-linoleic acid mixture without further supplement, and positive controls, *i.e.* corn-starch-linoleic acid mixture plus antioxidant compound of known potency, accompanied each experiment. One special advantage of the corn-starch-linoleic acid system over the usual procedures of measurement of antioxygenic activity is that it is carried out at room temperature up to 30°.

TABLE I

Antioxidant Activity of N,N-Dimethylaminoazobenzene (Butter Yellow)

Composition of assay mixture per gm	2nd day		3rd day		4th day		5th day		8th day		21st day	
	Butter yellow per gm	I No	Butter yellow per gm	I No	Butter yellow per gm	I No	Butter yellow per gm	I No	Butter yellow per gm	I No	Butter yellow per gm.	I No
	mg		*mg.*		*mg.*		*mg.*		*mg.*		*mg.*	
(1) 0.84 gm. corn-starch + 0.16 gm. linoleic acid		127.2		91.2		72.7		56.3				
(2) (1) + 0.6 mg. butter yellow	0.50	131.5	0.31	100.5	0.14	82 0	0.06	55.9				
(3) (1) + 1.0 mg. butter yellow	0.95	132.7					0.18	56 9	0.09	42.8		
(4) (1) + 2.5 mg. butter yellow		132.8					1.3	124.3	0.72	67.3	0.44	44.3
(5) (1) + 5.0 mg. butter yellow.		133.0					5.1	133.1	4.8	131.6	4.5	136.9

The results of experiments presented in Table I demonstrate that the antioxygenic activity of butter yellow is not to be neglected. To be sure, at the level (0.6 mg. per 1 gm. of the starch-linoleic acid mixture) at which butter yellow was employed in the previous studies as an indicator of rancidity, no definite inhibition of rancidity could be found. However, with higher doses the antioxygenic activity of butter yellow became distinctly manifest. To secure complete protection for the 21 days of the experimental period, 5.0 mg. of the dye per gm. of mixture were necessary, 2.5 mg. offering only moderate protection to the 21st day. When compared with the classical antioxidants, hydroquinone and α-tocopherol (Assay Mixtures 5, 7, and 9 of Table II), it is apparent that in this mixture the carcinogenic dye has an activity greater than that of α-tocopherol and about equal to that of hydroquinone.

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[1] "Linoleic acid, refined light," from the Glyco Products Company, Inc., Brooklyn, New York. The iodine number of the different lots varied from 126 to 140.

TABLE II

Activity of Butter Yellow in Conjunction with Other Antioxidants

Experiment	Composition of assay mixture per gm.	5th day		8th day		21st day	
		Butter yellow per gm	I No.	Butter yellow per gm	I No.	Butter yellow per gm	I No.
		mg.		*mg*		*mg.*	
A	(1) 0.84 gm. corn-starch + 0.16 gm. linoleic acid		61.4				
	(2) (1) + 0.6 mg. butter yellow	0.09	68.2				
B	(3) (1) + 1.0 " hydroquinone		97.3		53.5		40.2
	(4) (1) + 1.0 " " + 0.6 mg. butter yellow	0.55	118.4	0.26	88.1	0.07	40.5
C	(5) (1) + 2.0 mg. hydroquinone		104.5		65.8		43.8
	(6) (1) + 2.0 " " + 0.6 mg. butter yellow	0.54	135.8	0.56	127.2	0.50	124.8
D	(7) (1) + 2.0 mg. α-tocopherol		126.9		96.9		39.5
	(8) (1) + 2.0 " " + 0.6 mg. butter yellow	0.60	136.7	0.11	85.7	0.08	43.5
E	(9) (1) + 5.0 mg. α-tocopherol		116.4		110.9		35.8
	(10) (1) + 5.0 " " + 0.6 mg. butter yellow	0.59	128.1	0.27	110.9	0.08	45.7
F	(11) (1) + 0.01 cc. rice bran extract		108.1		55 1		40.4
	(12) (1) + 0.01 " " " " + 0.6 mg. butter yellow	0.47	132.2	0.25	113.7	0.08	51.1
G	(13) (1) + 0.05 cc. rice bran extract		99.9		70.4		44.8
	(14) (1) + 0.05 " " " " + 0.6 mg. butter yellow	0 59	134.1	0.55	120.2	0.60	124.0

In Table II data are presented on a study of the synergistic action of butter yellow with several typical antioxidants. Hydroquinone was selected as characteristic of the phenolic type, α-tocopherol as an inhibitol, and aqueous extract of rice bran as the most potent of natural antioxidants in sources of the vitamin B complex.

From these investigations it became apparent that while butter yellow acts synergistically with either hydroquinone or rice bran extract it does not do so with α-tocopherol. Whereas 0.6 mg. of butter yellow per gm. alone offers no protection to the oxidation of linoleic acid (Experiment A), in the presence of 2.0 mg. of hydroquinone per gm., which has little activity alone, complete protection is found at the 21st day of the experiment (Experiment C). Similarly the antioxygenic activity of rice bran extract is greatly enhanced by the addition of 0.6 mg. of butter yellow per gm. With 0.05 cc. of the rice bran extract per gm. the iodine number of the linoleic acid decreased to 108.1 by the 5th day; the addition of 0.6 mg. of butter yellow per gm. afforded a mixture with complete protection up to the 21st day (Experiment G) and according to previous studies (12) up to 5 months and longer. The combination of butter yellow and rice bran extract constitutes a remarkable example of antioxygenic synergism.

DISCUSSION

In the production of liver cancer by feeding rats butter yellow the highest incidence has always been obtained with a diet containing brown rice. The postulation that the water-soluble antioxidant of brown rice is responsible for this procarcinogenic action (7) must now be amplified with the newer knowledge that the antioxygenic activity of the factor (or factors) in brown rice is greatly enhanced by the presence of butter yellow. Butter yellow in conjunction with the antioxidants of the rice inhibits the oxidation of unsaturated fat and consequently preserves the carcinogenic dye so that it may be carried intact to the liver cells.

Sugiura and Rhoads (13) in an attempt to isolate the procarcinogenic factor of brown rice fed rats an ether extract of whole rice. Contrary to their expectations, this fraction decreased the incidence of liver cancer, a result which may now be attributed to the unsaturated fat content of the rice oil. The fact that the tocopherols present in such rice oil were without any procarcinogenic activity is in good accord with the *in vitro* experiments reported in this paper, showing the inability of α-tocopherol, with or without butter yellow, appreciably to retard the oxidation of linoleic acid.

Inhibition of Enzymatic Oxidation of Linoleic Acid

In the regulation of physiological oxidition prooxidants will undoubtedly play as important a rôle as antioxidants. The enzyme lipoxidase is a special example of factors which promote and accelerate the oxidation of unsaturated fat. Lipoxidase activity has been demonstrated in the past in a number of plant sources. Recently Hove (14) reported the presence of lipoxidase in mammalian tissue, in particular in aqueous extracts of minced stomach and liver of rats.

The inhibition of fat oxidation by antioxidants besides being a direct action consisting mainly in the prevention of peroxide formation might also result from inactivation and neutralization of prooxidant activities, such as for instance copper ions, etc. On the basis of these and similar considerations a number of antioxidants known to be effective in the corn-starch-linoleic acid mixture were studied concerning their effect on the oxidation of linoleic acid catalyzed by lipoxidase.

Experimental and Results

The effect of antioxidants on fat oxidation catalyzed by lipoxidase was studied by a measurement of the rate of disappearance of color from a linoleic acid-carotene solution. This method with minor variations has been used by a number of workers (15–17). The reaction mixture employed in this study was essentially that of Sumner and Sumner (15). The following components, in the order given, were introduced into a 500 cc. flask: 0.175 mg. of crystalline carotene (S. M. A. Corporation) in 5 cc. of acetone; 2.5 mg. of linoleic acid in 5 cc. of acetone, the antioxidant in 1 cc. of acetone, 5 cc. of phosphate buffer (pH 6.5); 100 cc. of distilled water and 0.2 to 0.5 cc. of enzyme preparation (2.5 per cent aqueous extract of ground defatted soy bean). With these proportions a stable clear yellow solution was obtained. The flask was agitated by a mechanical shaker which was halted at various intervals to permit the withdrawal of 10 cc. of the reaction mixture. This was quickly pipetted into 0.5 cc. of concentrated hydrochloric acid which inactivated the enzyme. The color of the mixture was determined by a Klett-Summerson photoelectric colorimeter with a blue filter (No. 42). The time required for 50 per cent decolorization served as an index of the rate of carotene destruction through the oxidized linoleic acid. The ratio of this value in a reaction mixture containing an antioxidant to that of a control (acetone blank) gave an expression of the inhibitory activity of the antioxidant. The results obtained with a number of compounds of known antioxidant activity are presented in Table III. Each value represents an average of three or four determinations. The amount of enzyme preparation used was such that the 50 per cent decolorization time fell between 200 and 400 seconds.

Of the compounds studied only α-tocopherol has shown appreciable inhibitory action. Approximately the same effect was had with 0.1 mg. of α-tocopherol as with 100 times as much hydroquinone or 50 times as much of the phenyl ether antioxidants. Butter yellow at a level of 0.1 mg. was not inhibitory; higher concentrations could not be tested because of interference by the color of the dye. The addition of rice bran extract to the reaction mixture produced stable emulsions which prevented colorimetric determination. With the exception of α-tocopherol, diphenylamine was the only other of the compounds tested that exerted a definite inhibition. The action of diphenylamine may be due not to an inhibition of the oxidation of linoleic acid and the consequent preservation of carotene but to a direct protection of the carotene by the amine. Williams, Bickoff, and Van Sandt (18) have found that of about 100 compounds tested diphenylamine was the best in preventing the oxidation of carotene when present in the stable medium of mineral oil.

It should be pointed out that, whereas for the corn-starch-linoleic acid and butter yellow mixture hydroquinone was found to be a satisfactory antioxidant and α-tocopherol a weak one (Table I), the reverse was true when they were used in the lipoxidase-linoleic acid-carotene mixture (Table III). This difference became even more distinct with the benzyl ether of hydroquinone in place of the free hydroquinone. Benzyl ether of hydroquinone and, to a lesser degree, methyl ether of hydroquinone, as well as *p*-hydroxydiphenyl ether and 4,4'-dihydroxydiphenyl ether, are excellent antioxidants when used in the linoleic acid-corn-starch system with and even without butter yellow,[2] but are much weaker antioxidants than α-tocopherol in the lipoxidase system.

TABLE III

Effect of Antioxidants on Enzymatic Oxidation of Linoleic Acid

Antioxidant	Ratio of 50 per cent decoloriza-tion time to that of control	Antioxidant	Ratio of 50 per cent decoloriza-tion time to that of control
1 mg. hydroquinone	1.4	0.04 mg. α-tocopherol	1.0
2 " "	1.3	0.1 " "	2.3
5 " "	1.5	0.4 " "	8.4
10 " "	2.4	5 " diphenylamine	3.9
5 " methyl ether of hydroquinone	1.8	5 " "	4.4
		0.1 " butter yellow	1.2
5 " benzyl ether of hydroquinone	1.6		
5 " *p*-hydroxydiphenyl ether	1.9		
5 " 4,4'-dihydroxydiphenyl ether	1.6		

SUMMARY

1. N,N-Dimethylaminoazobenzene (butter yellow) possesses considerable activity in retarding the autoxidation of linoleic acid.

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[2] Unpublished experiments in collaboration with M. B. Williamson (19).

2. Butter yellow acts synergistically in enhancing the antioxidant activity of rice bran extract or hydroquinone but is ineffective with α-tocopherol.

3. Of a number of antioxidants tested only α-tocopherol exerted any appreciable inhibition to the oxidation of linoleic acid (and carotene) catalyzed by the enzyme soy bean lipoxidase. Diphenylamine displayed a slight action, while hydroquinone, methyl and benzyl ethers of hydroquinone, diphenyl ethers, and butter yellow gave no inhibitory effect.

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THE DIRECT DETERMINATION OF 5-KETO-D-GLUCONIC ACID

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(Received for publication, April 3, 1944)

No methods have been available for the direct determination of 5-keto-D-gluconic acid in the presence of other sugars. Those employed thus far have depended on polarimetric changes and differences in the reduction of hot alkaline copper reagents. Stubbs, Lockwood, *et al.* (1) used the Shaffer-Hartmann reagent to determine this acid in fermentations, but the analysis was based on the absence of glucose and similar compounds. Iodine consumption in alkaline solution (which determines total aldose concentration) plus a total copper reduction method is not advisable, because 5-keto compounds react somewhat with alkaline hypoiodite.

We have found that the rate of reaction between 5-keto-D-gluconate and cold Benedict's reagent can be used to determine this sugar quantitatively in the presence of a variety of other compounds. Glucose, galactose, mannose, fructose, 2-keto-D-gluconic acid, and other common saccharides interfere only when present in high quantities. The presence of salts, likewise, can be tolerated in considerable concentration.

L-Ascorbic acid and its oxidation products, dehydroascorbic acid and 2,3-diketo-L-gulonic acid, as well as compounds of similar structure, also reduce cold copper reagents. They must be absent or removed in the determination of 5-keto-D-gluconic acid.

Procedure

Reagents—

1. Benedict's quantitative reagent, with modifications, as follows: copper sulfate pentahydrate, 18.0 gm.; sodium carbonate, anhydrous, 150.0 gm.; potassium citrate, 200.0 gm.; potassium thiocyanate, 125.0 gm.; potassium ferrocyanide, 5.0 ml. of a 5 per cent solution. The salts are dissolved in water and made up to 1 liter. The reagent should be allowed to stand for several days before use and then decanted from any precipitate which may arise.

2. Standard solution of 5-keto-D-gluconic acid. 2.5000 gm. of calcium 5-keto-D-gluconate (8.50 per cent calcium) are warmed slightly with 0.7500 gm. of oxalic acid dihydrate dissolved in 20 to 30 ml. of water. After the calcium oxalate is filtered off, the volume is made up to 50 ml. in a volumetric flask with water. From this standard, various dilutions can be made. The standard is stable for at least 5 to 6 weeks in the ice box.

3. Standard solution of sodium 5-keto-D-gluconate. This standard is made up the same as the acid standard, except that a neutralization with sodium bicarbonate precedes the dilution to 50 ml. The amount of bicarbonate should always be calculated accurately from the quantity of oxalic acid used in decomposing the calcium salt.

4. Comparison tubes. Test-tubes 5 by 1 inches are coated on the outside with white enamel up to a height of 2 inches from the bottom.

All solutions are brought to 25° in a constant temperature bath before use and are held at that temperature throughout the determination.

5 ml. of Benedict's reagent are placed in the comparison tubes and held in the temperature bath. Then 5 ml. of the standard (or solution to be tested) are pipetted into the reagent which is shaken to provide uniform agitation during this operation. The time necessary for the complete disappearance of the blue color is determined with a stop-watch, in which the starting time is taken as the first moment during which pipetting of solution into the reagent begins.

For maximum accuracy the time for pipetting the solution into the reagent should be between 15 and 20 seconds.

Results

The times required for the complete reduction of solutions containing various concentrations of 5-ketogluconate are given in Table I. When the times are plotted against concentration, Curve A of Fig. 1 is the result. From this curve the concentration of unknown solutions may be obtained by interpolating from the time of reduction of a known dilution.

The reduction times as shown in Table I can be duplicated within 5 seconds on duplicate samples from the same solution. The accuracy, of course, depends upon the operator's ability to see the disappearance of the blue copper color. For this reason it is necessary for each individual to establish his own curve to avoid errors in personal differences. Like all Benedict's reagent, each new solution must be standardized.

The reduction of 5-ketogluconic acid is little affected by the presence of other compounds of common origin. Glucose, which does not reduce the reagent in 24 hours, has no effect on the determination unless present in very large quantities. In Table II the action of glucose, as well as of some other monosaccharides, on the determination of 5-ketogluconic acid is shown. At a concentration of 50 mg. per ml. of glucose the time of reduction is hastened only 14 seconds from its previous value of 15 minutes and 4 seconds. Other monosaccharides, aside from fructose, have approximately the same effect as glucose and can be present in high concentrations without causing much difficulty. It will be noted, however, that fructose in amounts of 50 mg. per ml. causes an error which hastens the time of reduction sufficiently to give an error of 10 per cent in the figure for 5-ketogluconic acid. This effect, obviously, becomes less with lower concentrations of fructose; in 25 mg. per ml. the error is practically negligible. An explanation of the fructose anomaly cannot be given as yet.

TABLE I

Reduction of Cold Benedict's Reagent by 5-Ketogluconate

5-Ketogluconate calculated as calcium salt	Time of reduction	
mg. per ml.	*min.*	*sec.*
50.0	7	16
40.0	9	0
30.0	11	30
27.5	12	40
25.0	14	10
22.5	15	30
20.0	17	50
17.5	20	30
15.0	24	20
10.0	41	0

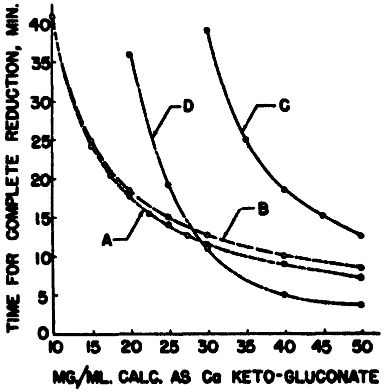

FIG. 1. The reduction time-concentration curves of 5-keto-D-gluconate (Curve A), 5-keto-D-gluconic acid (Curve B), 5-keto-D-gluconic acid contaminated with saccharic acid (Curve C), and an unknown compound (Curve D).

Special note should be made of 2-keto-D-gluconic acid[1] which because of its similarity to the 5-keto acid could be expected to lead to interference. The 2-keto acid, however, does not reduce the reagent, except on standing with it for long periods of time, nor does it affect the time of reduction of 5-ketogluconic acid. The data of Table II show this clearly. The reduction toward cold Benedict's reagent represents, therefore, a distinct difference between these two acids and can be used as a means of determining one in the presence of the other.

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[1] Supplied through the courtesy of Dr. C. L. Mehltretter, Northern Regional Laboratory.

Salts such as sodium chloride, sodium nitrate, sodium sulfate, sodium nitrite, and potassium chloride when present in concentrations as high as 50 mg. per ml. do not change the time of reduction of 5-ketogluconic acid and consequently introduce no error in the determination. This is particularly significant when solutions containing mineral acids are neutralized preparatory to the determination.

TABLE II

Effect of Glucose and Other Monosaccharides on Cold Reduction of 5-Ketogluconic Acid (25.0 Mg. per Ml.)

Sugar	Concentration of sugar	Time of reduction		Concentration* calculated from reduction
	mg per ml	*min*	*sec*	*mg. per ml*
Glucose	50	14	50	25.5
Mannose	50	14	32	26.0
Galactose	50	14	50	25.5
Fructose	50	13	40	27.5
"	25	14	50	25.5
2-Ketogluconate	50	15	0	25.0
		15	4	25.0

* Values referred to Curve B, Fig. 1.

The reagent as here employed is not reduced by the common monosaccharides and 2-keto-D-gluconic acid nor by such molecules as uronic acids, pyruvic acid, levulinic acid, formic acid, formaldehyde, acetaldehyde, and amino acids. In the presence of these 5-ketogluconic acid can be determined readily by diluting the neutralized solutions to a standard volume and obtaining the concentrations from Curve A.

There are, of course, compounds other than 5-ketogluconic acid which show a marked reducing action toward cold Benedict's reagent. Those of which we are certain are enediols, such as L-ascorbic acid, and their oxidation products. Of these dehydroascorbic acid and 2,3-diketo-L-gulonic acid reduce readily. The presence of compounds of this nature can be detected easily by suitable qualitative tests (2–4). The enediols can be oxidized with iodine without affecting 5-ketogluconic acid; but the resulting oxidation products cannot be obviated quite so readily. They are susceptible to heat and their concentration can be determined in the presence of 5-keto acid by a procedure which will be reported in another publication.

The determination of 5-ketogluconic acid in the presence of compounds like dehydroascorbic acid is naturally obscured by their reducing action. Nevertheless, we have found the reduction times of such composite solutions to be of considerable value in following the progress of certain oxidations. For instance, in the nitric acid oxidation of gluconic acid (and glucose) progress of the reaction was followed by removing 5 ml. of the solution at frequent intervals, neutralizing with sodium bicarbonate, diluting to 50 ml., and determining the reduction time. In Table III some typical results are shown for gluconic acid. The oxidation for glucose runs a similar course.

It will be noted that two curves, A and B, are given in Fig. 1. Curve A is the time-concentration curve for solutions which have been neutralized before the determination, while Curve B represents the curve for solutions of the free acid which are employed without prior neutralization. Curve A is used for complex solutions in which one does not effect a preliminary isolation of the acid, as in the oxidation of glucose by microorganisms or by nitric acid. Curve B, on the other hand, has been most useful in analyzing the salts of acids which are isolated from reaction mixtures. Almost invariably we have isolated our acids as the calcium salts. These are decomposed by oxalic acid and allowed to remain as the free acids, because the free acids are much more stable in acid solution than in neutral or alkaline conditions. We have kept refrigerated solutions of 5-ketogluconic acid for months without deterioration.

TABLE III

Reduction of Solutions Obtained from Nitric Acid Oxidation of D-*Gluconic Acid*

Reaction sampled	Time of reduction
hrs.	
16	No reduction
18	3.75 hrs.
19.5	17 min. 30 sec.
20.25	12 " 30 "
21.75	9 " 45 "
23	6 " 0 "
40	3 " 0 "
91	1 " 5 "

The differences in Curves A and B arise from the fact that the reduction velocity of the cold copper reagent is intimately associated with the concentration of sodium carbonate. Hence, when a solution of the free acid is added, part of the sodium carbonate is removed through the ensuing neutralization. The decrease in alkalinity lengthens the time of reduction.

The two curves meet obviously when the concentration of acid is small, and a negligible diminution in alkali results.

The following result shows the effect of sodium carbonate concentration on the reduction time of a solution containing 5-ketogluconic acid. The reduction time with the normal reagent was 8 minutes, 30 seconds, which corresponds to a concentration of 50 mg. per ml. When in addition 25 mg. of sodium carbonate per ml. were added to the reagent, the time was reduced to 7 minutes, 33 seconds. Because of this dependence on alkalinity we have always kept constant the ratio of oxalic acid to calcium salt when making up solutions to be run according to Curve B.

In addition to giving quantitative results the time reduction curve of a compound can also be used as a qualitative means of identification. In a preparation in which 5-ketogluconic acid is solely the reducer present, the curve will be similar to, or identical with Curve A or B, depending on the conditions employed. An example of this situation is given by Curve C in Fig. 1 in which a calcium salt contaminated with saccharate was run. Curve C fits the portion of Curve B between concentrations of 10 to 30 mg. per ml. On the other hand, a calcium salt isolated from the oxidation of galactose gave a curve represented by Curve D. The nature of this salt is still being investigated. As yet it is impossible to draw a definite conclusion regarding keto acids other than 5-ketogluconic, but we feel safe in assuming that their reduction curves will not be homologous with Curve A or B.

SUMMARY

A method for the determination of 5-keto-D-gluconic acid has been given in which the rate of reaction with a modified Benedict's reagent at 25° is employed. D-Glucose, 2-keto-D-gluconic acid, and other monosaccharides do not interfere with the determination, nor do sodium chloride, sodium sulfate, sodium nitrite, and potassium chloride.

The reagent is not reduced by formaldehyde, acetaldehyde, formic acid, pyruvic acid, levulinic acid, and uronic acids.

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AMINO ACID NITROGEN CHANGES IN SHOCK

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(Received for publication, April 12, 1944)

In 1943 Engel, Winton, and Long (1) reported profound changes in the amino acid nitrogen of blood in hemorrhagic shock. According to these workers the changes were due primarily to a decrease in hepatic function resulting from anoxia. Secondary effects of anoxia on the peripheral tissues caused an enhanced rate of protein breakdown there and contributed to the picture of rising amino acid nitrogen. In subsequent papers by Russell, Long, and Engel (2) and by Engel, Harrison, and Long (3) it was shown that there was an increase in peripheral protein metabolism in animals following hemorrhage and that the products of this metabolism accumulated either because they were not circulating through the liver sufficiently rapidly or because with continued anoxia the hepatic parenchyma was damaged and could not dispose of the amino acid.

In the present communication data are presented which indicate that anoxic peripheral tissues contribute amino acids to blood, and that this may lead to a general elevation of the plasma amino acid level.

Material and Methods

Dogs were anesthetized by sodium pentobarbital, and shock was produced by the application of pneumatic pressure cuffs to both hind limbs. The cuffs were left in position for a period of 5 hours and the pressure in the cuffs was maintained at about 230 mm. of Hg. Controls were anesthetized for the same length of time as the test animals. On removal of the cuffs the animals went into shock. The blood samples studied were a brachial vein sample obtained before anesthesia and several postanesthetic samples from femoral vein, femoral artery, jugular vein, and sometimes carotid artery.

Shock was produced in rats by clamping the two hind legs (Haist and Hamilton (4)). 3 to 4 hours after the clamps were removed, the animals were killed by stunning and a blood sample removed from the abdominal aorta. Control animals were fasted for the same period as the test animals but did not have their legs clamped.

All amino acid determinations were carried out on tungstic acid filtrates. Danielson's (5) modification of the Folin colorimetric method was employed for the majority of the determinations (studies of fourteen dogs and 58 rats). The method was adapted to the photelometer, with a Cenco No. 1

blue filter (transmitting light of wave-length 400 mμ). Since the dye used in the reaction is not entirely specific for amino acids, a comparison of this method with the ninhydrin manometric method of Van Slyke *et al.* (6) was attempted (studies on four dogs).

For the majority of the dogs of this series the non-protein nitrogen of the plasma was also determined. The micro-Kjeldahl technique was employed. These data are not set forth in detail, since non-protein nitrogen changes in shock have been described so often by others. However, they deserve a brief reference, since they add somewhat to the general picture.

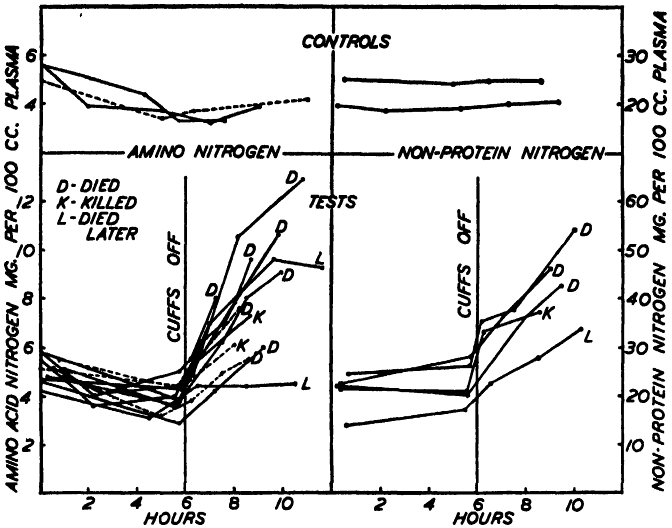

FIG. 1. Amino acid nitrogen (left) and non-protein nitrogen (right) changes for different control dogs (upper) and those in shock (lower). For amino nitrogen, broken lines indicate manometric, and solid lines colorimetric determinations.

Results

Changes in Control Dogs—Crismon *et al.* (7) found that pentobarbital produced a mean lowering of 1.7 (±0.3) mg. per cent in the blood amino acid nitrogen of dogs. Our findings are in agreement (Fig. 1). The amino acids fell rapidly at first, then more slowly for a period of from 5 to 8 hours. After that there was a tendency to return toward normal, although in one dog the amino acid nitrogen values were below normal even after 11 hours of anesthesia.

It was not deemed necessary to follow more than three dogs for prolonged periods of anesthesia, since the initial 5 hour period of cuff application in the test animals forms an anesthetic control in itself. The mean lowering of plasma amino acid nitrogen produced by 5 hours of anesthesia in twenty-five dogs was 1.1 mg. per cent. The mean preanesthetic value for these dogs was 4.9 mg. per cent.

That the anesthetic alone has little or no effect on the plasma non-protein nitrogen is shown in Fig. 1.

Changes in Dogs in Shock—Changes in plasma amino acid nitrogen and non-protein nitrogen values in dogs in shock are also shown in Fig. 1. In both cases the nitrogen values rise precipitously after removal of the pressure cuffs.

The general rise in amino acid nitrogen for eight dogs, as shown by jugular vein plasma, amounts to an increase of 68.6 per cent over preanesthetic values, or 3.6 mg. per 100 cc. The local rise, as shown by femoral vein plasma, is more than 100 per cent or 5.5 mg. per 100 cc. Considering the depression in amino acid level produced by the anesthetic (1.1 mg. per cent), it is evident that the absolute increase after removal of the cuffs is actually greater than these figures indicate.

The general picture of a progressively rising plasma amino acid level is seen with both manometric and colorimetric procedures. However, the limited data available with the manometric technique indicate that the rise (particularly in femoral vein plasma) may be less marked than the above results would suggest. It is possible that some other material (ammonia or uric acid?) capable of reacting with sodium naphthoquinone-4-sulfonate may contribute to the terminal picture when the colorimetric method is used (see Fig. 1).

One source of the amino acids in this type of shock is evident from a comparison of the blood samples from jugular vein, carotid artery, femoral vein, and femoral artery. The data are given in Table I and summarized in the histogram of Fig. 2. Femoral vein values are consistently the highest, while there is little difference in the plasma amino acid values of jugular vein, carotid artery, and femoral artery.

Engel, Winton, and Long have correlated the rising blood amino acids in hemorrhagic shock with a falling blood pressure, and suggest that the low blood pressure will produce an early anoxia of the liver and consequent impairment in the deamination processes. In the light of these findings we have considered our data in relation to the blood pressures. Amino acid nitrogen values and concomitant carotid blood pressures for eleven dogs in shock are shown in Fig. 3, *A*. The relationship between the falling blood pressure and rising amino acids is evident. However, we feel that a significant rise in the amino acid level of the blood generally may occur before the blood pressure has reached a critical level. In Fig. 3, *B*, for example, the blood pressure fell to 85 mm. of Hg for about 1 hour, then returned to normal, and the animal lived for 2 days. Nevertheless, the amino acids of the plasma rose from 4.1 to 9.6 mg. per cent with a blood pressure which does not seem to be critical. Again, in a series of cross-circulation experiments, significant increases in the amino acids of control animals were sometimes observed with mean arterial blood pressures of 120 to 140 mm. of Hg (Fig. 3, *C*). This was true also of the non-protein nitrogen of

TABLE I

Amino Acid Nitrogen in Mg. per 100 Cc. of Plasma

Dog No.	Preanesthetic; brachial vein	Before removal of cuffs		Shock, after removal of cuffs											
				0–60 min.				60–120 min.				120 min. until death			
		Jugular vein	Carotid artery	Jugular vein	Carotid artery	Femoral vein	Femoral artery	Jugular vein	Carotid artery	Femoral vein	Femoral artery	Jugular vein	Carotid artery	Femoral vein	Femoral artery
143	4.2	3.1		4.4		5.7	3.9	4.4		6.1		4.5		5.7	4.0
152	5.8	3.8		6.2		8.9	5.8	8.0		11.1	6.8	9.1		13.0	9.5
153	4.7	4.3	4.4	5.3	5.1	5.8						7.3	7.0	7.0	6.7
154	4.6	2.9	2.8	4.2	4.3	6.1	4.5	6.0	6.0	8.3	6.4				
176	5.1	4.4	7.0	7.0				8.0				9.6			
178	5.5	3.9		6.4				7.8				9.6			
183	4.8	3.6		5.0		5.1	5.8	6.5		10.0	7.0	10.6		13.0	9.6
192	5.2	4.0		3.9			4.0	5.1		9.3	4.8	6.0		12.8	6.4
199	6.0	5.5		5.6				8.3							
204	6.2	4.3	4.1					7.6	6.6	9.3	6.8				
205	5.1	4.4	4.3	5.2	5.5	5.8	5.1							12.6	8.9
215	5.1	3.5		4.9		5.1	3.5	10.5		11.6	4.3	12.8		10.0	3.3

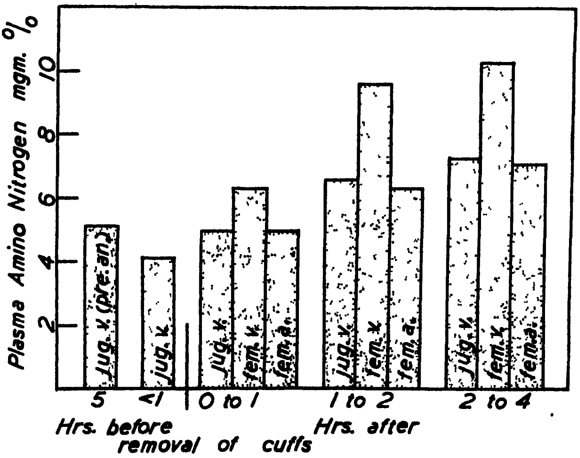

FIG. 2. Average amino acid nitrogen values for five dogs

the plasma. In these cross-circulation control experiments the only tissue which might have been seriously affected was the blood itself. Further, it seems suggestive that amino acid and non-protein nitrogen changes were evident in a series of dogs subjected to the usual shock-producing procedure but given an early transfusion (plasma, isinglass, or polyvinyl alcohol) so that the blood pressure did not fall to shock levels (Fig. 3, *D*).

These findings suggest that the rise in amino acid nitrogen is not always related to the falling blood pressure.

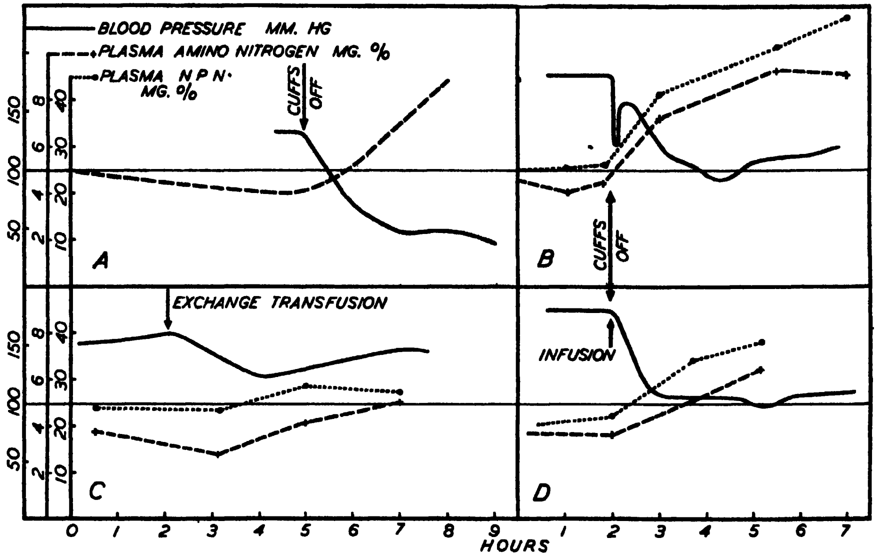

FIG. 3. Relationship between amino acid nitrogen and blood pressure in shock. *A*, regression lines for eleven dogs. *B*, Dog 176; shock produced in the usual manner; lived 2 days beyond the experiment. *C*, exchange transfusion control, Dog 244, showing nitrogen elevation with relatively high blood pressure. *D*, Dog 167, infused with a 4 per cent solution of polyvinyl alcohol in normal saline (40 cc. per kilo of body weight); dog survived.

Plasma Amino Acids in Rats—The changes in plasma amino acid nitrogen in unanesthetized rats are summarized in Fig. 4. A significant elevation in the amino acid nitrogen of the plasma is evident. However, the actual increase is less for the rat than for the dog (on the average, 2.2 mg. per cent as compared to 3.6 mg. per cent).

Haist and Hamilton, studying changes in the liver glycogen storage, found a failure of storage in the rat in shock but a restoration of the process when the limbs were reclamped. The reclamping procedure prevented death in our animals, but did not change the level of amino nitrogen within 4 hours (Fig. 4). At the end of that time the levels were still high. If it is assumed that the rise in amino acids is due either directly or indirectly to a failure of the deamination processes of the liver, then it follows that there is not a rapid recovery of the ability of liver to deaminate amino acids.

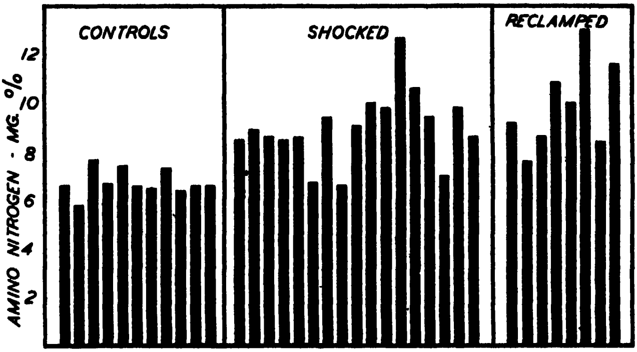

FIG. 4. Plasma amino nitrogen values for control, shock, and "reclamped" rats. In the latter group the clamps were left off for 2 hours; then the legs were reclamped and the plasma samples were taken 4 hours later.

DISCUSSION

The amino acid nitrogen of the blood has a value which is surprisingly constant under many changing physiological conditions. Injections of pentobarbital, insulin, epinephrine, and potassium chloride have been shown to produce a slight lowering (Crismon *et al.*). Injections of amino acids produce a rapid rise in the blood amino acid level, but this returns to normal within a few hours, owing to increased excretion of amino acids or their conversion to urea in the liver (Bollman, Mann, and Magath (8)). Consequently it has been suggested that the pronounced elevations seen in traumatic shock (Lurje (9)) and in hemorrhagic shock (Engel, Winton, and Long) may give a clue to some fundamental feature of the shock syndrome. The rise evident in shock would seem to indicate that the amino acids are being produced in relatively high concentrations or that the deaminating functions of liver, or removal by the kidneys, are seriously interfered with. In our dogs there is little or no excretion of urine after removal of the cuffs.

Both groups mentioned above have stressed the probability of liver damage in shock. In the case of hemorrhagic shock, Engel, Winton, and Long have shown that the rise in amino nitrogen in the rat occurs only after the blood pressure has fallen to 80 mm. of Hg, and suggest that the low blood pressure will produce an early anoxia of liver and consequent impairment in the deamination processes.

In the type of shock which we have been studying, the data for dogs show clearly that anoxic peripheral tissue contributes amino acids, as well as other non-protein nitrogenous constituents, to the blood. It seems possible that the non-protein and amino acid nitrogen coming from the injured limbs will account for the initial general rise noted. It is probable that other tissues become somewhat anoxic in the terminal stages of shock, and may add to the picture of rising blood nitrogen. Initially, however, in these experiments the rise in amino acid nitrogen of the shocked dog seems to be due to processes going on within the tissues of the injured limbs. Our data show that a general rise may occur before the blood pressure falls to 100 mm. of Hg. This blood pressure does not seem low enough to produce a generalized tissue anoxia, though an anoxia of certain peripheral tissues may be present. It is appreciated that following hemorrhage any effects of anoxia may not be evident immediately, whereas, in the type of shock we are studying, materials from tissues subjected to prolonged anoxia may be poured into the circulation immediately upon release of the cuffs.

The results of the reclamping experiments in the rats suggest that the liver function is altered in these animals. We would expect the reclamping (which cuts off the blood supply from the injured limbs) to prevent further contribution of amino acids to the blood. If the deaminating processes were unaffected, the amino acid nitrogen level of the blood should soon return to normal after the limbs are reclamped. This does not occur in 4 hours.

These findings on the rat confirm the theory that the liver function is altered in shock. It is of interest to note, however, that despite the fact that the blood amino acid levels remain high the animals begin to recover during the 4 hour "reclamped" period. It would appear therefore that certain other processes are more fundamental than those responsible for the elevation of the blood amino acid nitrogen level.

SUMMARY

Dogs in shock produced by pressure cuffs showed a rise in the plasma amino nitrogen of 3 to 5 mg. per cent.

In the control animals the plasma amino nitrogen was reduced 1 to 2 mg. per cent.

The primary rise in amino acid values in dogs in shock results from processes going on in the tissues of the injured limbs. Significant changes may occur before the blood pressure has fallen to shock levels.

The plasma amino nitrogen of the rat in shock is, on the average, 2 mg. per cent higher than that of the normal animal.

In both the dogs and rats, suitable treatment (early transfusions or reclamping of the injured limbs) may bring about recovery before the amino acid level shows any tendency to return to normal.

It is a pleasure to thank Professor C. H. Best, for his interest and help in this work.

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THE EFFECT OF PARATHYROID EXTRACT UPON THE DISTRIBUTION, RETENTION, AND EXCRETION OF LABELED PHOSPHORUS*

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(Received for publication, April 20, 1944)

Various views have been presented in regard to the site and the mechanism of action of parathyroid hormone. In 1929, Albright and Ellsworth (1) advanced the view that changes in the calcium metabolism, including changes in the bone, are dependent upon preceding changes in the phosphorus metabolism. After the administration of parathyroid extract to patients, Albright and Ellsworth (1) observed an immediate increase in the urinary excretion of phosphorus. They suggested that some change occurs in the blood equilibria as a result of parathyroid extract administration which makes necessary an increase in phosphorus excretion. On the basis of these observations (1), and additional investigations (2–4), Ellsworth (5) suggested that parathyroid hormone acts by lowering the renal threshold for phosphorus. As a result of recent studies of the action of parathyroid extract on nephrectomized rats, Ingalls, Donaldson, and Albright (6) suggest that the hormone acts on phosphate metabolism in some way which not only increases the excretion of phosphates in the urine, but also produces certain bone changes directly.

In 1932, Selye (7) advanced the theory that parathyroid hormone directly stimulates the formation of osteoclasts, whereby calcium is released to the blood. In support of this theory, Collip, Pugsley, Selye, and Thomson (8) observed that parathyroid extract produced osteoclastic resorption of bone in bilaterally nephrectomized rats, thus indicating a direct action of parathyroid hormone on the bone, independent of any action it may have on the kidney. Likewise, McJunkin, Tweedy, and McNamara (9) observed that the administration of parathyroid extract to nephrectomized rats caused a pronounced resorption of bone. However, they were unable to detect a rise in the serum calcium of the nephrectomized rat after the injection of massive doses of parathyroid extract. In previous experiments, Tweedy, Templeton, and McJunkin (10) were unable to observe a rise in the serum calcium of the nephrectomized dog after the injection of massive doses of parathyroid extract, despite the fact that the serum calcium continued to rise in the nephrectomized dog in which the action of parathyroid extract was initiated before nephrectomy. They concluded that the kidneys play a dominant rôle in the action of parathyroid hormone. After a study of the action of parathyroid extract on the serum calcium and the serum inorganic phosphate of nephrectomized rats, cats, and dogs, Neufeld and Collip (11) concluded that the results of their experiments did not suggest a direct action of parathyroid hormone on the bone, but rather a direct action on the kidneys whereby the excretion of phosphates is increased.

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* An abstract of a portion of the data reported here has been published (Campbell, W. W., and Tweedy, W. R., *Federation Proc.*, **1**, pt. 2, 104 (1942)).

Although interest has been centered chiefly on the kidneys and the bones as the sites of parathyroid hormone action, the liver has not escaped consideration as a possible site of action. In 1932, Greenberg (12) and Nitzescu (13), independently, observed that acute intoxication of the liver with elementary P practically obliterated the serum calcium-raising effect of parathyroid extract in the dog. After further studies on the effects of liver poisons, Greenberg (14) concluded that the deleterious effect of elementary P on the calcium-raising effect of parathyroid extract did not appear to be through an injurious action on the bone cells. More recently, Lederer and Crandall (15) observed a decreased effectiveness of parathyroid extract in mobilizing calcium in Eck fistula dogs. They concluded that these animals either suffer from a calcium deficiency which is secondary to a decrease in bile secretion, or that the observed effects are due to some endogenous relation of the liver to calcium metabolism.

The data obtained in this investigation indicate that parathyroid extract affects the distribution, retention, and excretion of radiophosphorus by producing changes in the phosphorus metabolism of the bones, the liver, and the kidneys.

EXPERIMENTAL

The young adult rats, which were used in these experiments (Table II), were reared on a diet of Purina fox chow, supplemented by greens and meat scraps twice weekly. In most instances the paired animals were not litter mates, but were of approximately the same age and weight.

The experimental animal of each pair (Table II) received a subcutaneous injection of 5 ml. (500 Hansen units) of parathyroid extract (Lilly). 1 hour later each animal of the pair was injected intraperitoneally with 0.5 ml. of a solution of 7 mg. of P (as Na_2HPO_4), containing 10 to 15 microcuries.

After the administration of the $Na_2HP^*O_4$, the experimental animal and its control were placed in separate wire bottom cages over urine-feces separators (16), and given access to food and water. At the end of the desired period, measured from the time of the administration of the labeled P, each animal was anesthetized, and sacrificed by drawing blood as completely as possible.

4 to 5 ml. of the animal's blood were transferred to a tared 10 ml. Coors porcelain ashing capsule, and rapidly weighed. The desired tissues were then dissected out and placed in stoppered weighing bottles. Weighed amounts of the tissues were transferred to 10 ml. ashing capsules, and these samples, together with the blood and the collections of urine and feces, were dried overnight in an electric oven, after which the samples were ashed with $Mg(NO_3)_2$ in an electric muffle at 450–500°. The ash was dissolved in HCl and either the whole solution or an aliquot was slowly evaporated to nearly dryness. Water was added and the evaporation carried to dryness to remove excess HCl.

The radioactivity of each test sample was measured by means of a Lauritsen electroscope by reference to a standard sample which represented a suitable aliquot of the original $Na_2HP^*O_4$.

Results

Blood—The action of parathyroid extract was not evident in the blood. With the exception of the first pair of animals (Table I) no other instance was encountered in which the P^{32} content of the blood of an experimental animal differed appreciably from that of its control.

Distribution and Retention—The two pairs of animals which were sacrificed 1 hour after the administration of the labeled phosphate (Table I) were male litter mates. When the radioactivities of their organs are compared on a gm. basis, the accumulation of P^{32} in the liver of the experimental animal Rat E-1 does not appear to have been affected by parathyroid extract, but on the same basis of comparison there appears to be slightly more P^{32} in the kidneys of Rat E-1 than in the kidneys of its control. On the other hand, when the activities of whole organs are compared, it will be seen that the experimental animal's liver contained 16.89 per cent of the administered radiophosphorus as compared with 13.15 per cent in the liver of its control, while the kidneys of the two animals contained exactly the same amount of the administered radiophosphorus. This is the only instance encountered in our experiments, however, in which a comparison of activities on a gm. basis only would have led to an erroneous conclusion concerning the accumulation of radiophosphorus in the whole organ.

In the case of the second pair of animals that was sacrificed at the 1 hour interval (Table I), as well as the three pairs which were sacrificed at the 2 hour interval, a comparison of activities, on either the gm. basis or per whole organ, shows that the liver and the kidneys of the hormone-treated animals retained larger amounts of radiophosphorus than the corresponding organs of their controls.

An examination of the liver and the kidneys of the hormone-treated animals, sacrificed at the various intervals throughout the 48 hour experimental period, generally revealed a higher specific content of radiophosphorus (retention) than the corresponding organs of the controls (Table II). However, the femurs of the hormone-treated animals exhibited no such constancy in the relation of their specific content of radiophosphorus to that of their respective controls.

1 hour after the administration of the labeled phosphate, the femurs of the hormone-treated animal Rat E-1 (Table I) contained considerably less P^{32} than the femurs of its control, while the femurs of the hormone-treated animal Rat E-2 contained only slightly less P^{32} than its control. At the 2 hour interval the femurs of the hormone-treated animals, Rats E-3 and E-4, showed a higher specific content of P^{32} than their respective controls, while the femurs of the experimental animals which were sacrificed at the 4 and 8 hour intervals (Table II) exhibited about the same specific content of P^{32} as that shown by their respective controls. After 8 hours the specific content of P^{32} in the femurs of the hormone-treated animals appears to have decreased markedly. In the animals sacrificed at the 18 and 24 hour interval (Table II), the femurs of those receiving parathyroid extract show a lower specific content of radiophosphorus than those of the untreated rats. At the end of the 48 hour experimental period, the specific content of radiophosphorus in the femurs of treated and the untreated rats is about the same.

TABLE I

Effect of Parathyroid Extract upon Distribution, Retention, and Excretion of Labeled Phosphorus

All values are expressed as per cent of the administered radiophosphorus recovered. E represents experimental animal, C control.

Rat No	Weight	Time after injection of P^{32}	Blood	Liver		Muscle	Femurs		Kidneys		Stomach, small intestine, and contents (whole)	Large intestine and contents (whole)
	gm	*hrs*	*per gm*	*per whole organ*	*per gm*	*per gm*	*per whole bone*	*per gm*	*per whole organ*	*per gm*		
E-1	204	1	0.19	16.89	1.42	0.12	3.11	2.55	2.19	1.16	6.82	2.32
C-1	204	1	0.25	13.15	1.41	0.13	3.39	2.92	2.19	1.10	5.60	2.21
E-2	202	1	0.22	14.79	1.80	0.11	3.38	2.80	2.65	1.49	6.43	2.32
C-2	204	1	0.24	13.54	1.41	0.14	3.45	2.90	2.09	1.11	6.43	2.31
E-3	196	2	0.22	10.67	1.77	0.17	1.16	1.08	2.04	1.32	3.11	2.91
C-3	196	2	0.19	8.72	1.34	0.19	1.03	0.87	1.70	1.06	3.27	2.92
E-4	183	2	0.17	9.03	1.40	0.14	1.17	1.07	1.85	1.18	4.48	1.31
C-4	175	2	0.19	8.41	1.01	0.18	0.97	0.85	1.45	0.97	4.47	1.48
E-5	247	2	0.17	13.14	1.56	0.18	1.04	0.75	2.03	0.99	4.57	1.66
C-5	255	2	0.16	9.89	1.29	0.08	0.97	0.69	1.94	0.93	4.74	1.90

TABLE II

Effect of Parathyroid Extract upon Distribution, Retention, and Excretion of Labeled Phosphorus

The experimental and the control animals are designated by the letters E and C, respectively.

No. of pairs	Weight of animal		Time after administration of P^{32}	Per cent* of administered radiophosphorus per gm of tissue										Per cent* of administered radiophosphorus recovered							
				Blood		Liver		Skeletal muscle		Femurs		Kidneys		Urine		Feces		Stomach, small intestine, and contents		Large intestine and contents	
	E	C		E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C
			hrs.																		
2	203	204	1	0.21	0.25	1.61	1.41	0.12	0.14	2.68	2 91	1.33	1.10	2.6	0.9			6.63	6.02	2.32	2.26
	±1	±0		±0.02	±0.01	±0.19	±0.00	±0.01	±0 01	±0.13	±0.01	±0.16	±0.01					±0.20	±0.42	±0.00	±0.05
3	209	209	2	0.19	0.18	1.54	1.21	0.16	0.15	0.97	0.80	1.16	0.99					4.04	5.14	2.15	1.52
	±34	±41		±0.03	±0.02	±0.20	±0.18	±0.02	±0 06	±0.19	±0.10	±0.17	±0.07					±0.82	±0.99	±0.80	±0.25
2	242	247	4	0.16	0.16	1.25	1.10	0.16	0.16	0.72	0.69	0.87	0.77	34.2	8.6	0.32	0.02	5.27	5.17	1.58	1.34
	±12	±7		±0.01	±0.01	±0.18	±0.06	±0.06	±0.01	±0.23	±0.31	±0.02	±0.02					±0.45	±0.79	±0.36	±0.02
2	189	189	8	0.14	0.14	0.96	0.80	0.21	0.20	1.03	0.98	0.60	0.50	23.4	20.9	1.67	0.22	3.13	2.95	1.15	1.40
	±4	±0		±0.03	±0.00	±0.08	±0.03	±0.01	±0.00	±0.06	±0.07	±0.03	±0.04					±0.27	±0.02	±0.05	±0.41
3	188	190	18	0.14	0.14	0.96	0.87	0.21	0.20	1.42	1.65	0.76	0.60	23.2	17.2	2.17	4.32	4.01	5.15	1.49	2.09
	±11	±12		±0.03	±0.02	±0.28	±0.15	±0.00	±0.01	±0.43	±0.57	±0.34	±0.09	±1.6	±4.2	±0.93	±1.82	±0.18	±0.56	±0.28	±0.75
5	199	205	24	0.12	0.11	0.78	0.67	0.22	0.21	1.30	1.65	0.59	0.52	15.3	11.7	2.98	4.41	5.11	4.81	1.27	1.29
	±19	±34		±0.03	±0.02	±0.14	±0.07	±0.07	±0 07	±0.53	±0.65	±0.12	±0.05	±6.1	±4.3	±2.23	±2.41	±3.00	±1.70	±0.40	±0.28
3	218	216	48	0.09	0.09	0.79	0.56	0.24	0.21	0.52	0.57	0.53	0.47	3.7†	5.0†	1.87†	2.11†	3.04	3.73	2.02	2.11
	±28	±19		±0.00	±0.01	±0.11	±0.05	±0.01	±0.01	±0.08	±0.05	±0.05	±0.05	±1.9	±1.6	±0.50	±0.11	±0.09	±0.04	±0.05	±0.04

* Mean per cent. The measure of variability for three or more values is the standard deviation from the mean.

† The first 24 hour excretion values are included in the average values shown for the animals sacrificed at the 24 hour interval.

The administration of parathyroid extract appears to have exerted no effect upon the movement of labeled phosphate into and out of the skeletal muscles (Table II). The variations from the average values at the various intervals cannot be ascribed to the action of exogenous parathyroid hormone, since the variations are as marked and occur as frequently in the control as in the hormone-treated series.

Urinary Excretion—Urine samples were obtained from one pair of animals (designated Rats E-2 and C-2, Table I) as early as 1 hour after the administration of labeled phosphate (Table II). After these animals were sacrificed, the abdominal cavity of each was opened and a ligature passed around the urethral orifice of the bladder. The urine sample was withdrawn through the wall of the bladder and measured by means of a tuberculin syringe. From the bladders of Rat E-2 and its control Rat C-2, 0.3 and 0.5 ml. amounts of urine, respectively, were transferred to ashing capsules and the activities determined in the manner previously described. The urine of the hormone-treated animal Rat E-2 was found to contain 2.6 per cent of the administered labeled phosphate, while that of its control, Rat C-2, contained only 0.9 per cent. Unfortunately this was the only instance in this series of experiments (Table II) in which a sample of urine was obtained as early as 1 hour after the administration of labeled phosphate. Subsequent collections of urine were made in the usual way by collection in a metabolism cage over a urine-feces separator.

At the 4 hour interval the hormone-treated animal of one of the two pairs of animals shown in Table II excreted 34.2 per cent of the administered phosphate, while its control excreted only 8.6 per cent. At the 8 hour interval the pooled urines of two hormone-treated animals (Table II) showed an average excretion of 23.4 per cent of the administered phosphate, while the pooled urines of their controls showed an average excretion of 20.9 per cent.

The percentages shown for the urinary excretion of P^{32} at the 18 hour interval (Table II) are the average values of the separate collections from three hormone-treated animals and three controls. The values obtained for the 24 hour collections of urine of the animals sacrificed at 48 hours are included in the average values shown for the animals sacrificed at the 24 hour interval.

Fecal Excretion—In both the experimental and the control group, great variation occurred in the amount of radiophosphorus which appeared in the collected feces (Table II). However, when the per cent of radiophosphorus recovered from the feces of a hormone-treated animal was compared with that of its respective control, nine of the eleven hormone-treated animals, from which feces were collected at the 18 or 24 hour interval, showed smaller amounts of radiophosphorus in their feces than their respective controls.

Separate recoveries of radiophosphorus from the intestines and their contents are not given in Table II. In several instances separate recoveries were made, but a comparison of activities of the intestinal tissues revealed no significant difference which could be attributed to the action of parathyroid extract. However, it was found that the sum of the percentages of radiophosphorus recovered from the gastrointestinal tract, its contents, and the collected feces served as an indication of the amount of radiophosphorus passing out by the fecal route. The percentage value so obtained was smaller in the case of each of the nine experimental animals mentioned above than for their respective controls.

DISCUSSION

The results of the present investigation indicate that the administration of a sufficient amount of parathyroid extract to the normal rat definitely accelerates the uptake of radiophosphorus by the liver. Although the difference between the specific content of radiophosphorus in the livers of treated and the untreated rats at various intervals up to 48 hours was variable, and not strikingly large, calculations based on the data in Table II show the average specific content of radiophosphorus in the livers of treated rats to be 20 per cent higher than that of the untreated animals. This observation is of particular interest, since it suggests a direct action of parathyroid hormone on phosphorus metabolism in the liver.

Parathyroid extract did not appear to accelerate the uptake or promote the retention of radiophosphorus in the femurs. The observed differences in the initial uptake and specific content of radiophosphorus by the femurs of treated and untreated rats up to the 8 hour interval (Table II) do not indicate an immediate action of parathyroid extract on the bones. However, between the 8 and the 18 hour interval (Table II) the femurs of treated animals began to lose their radiophosphorus more rapidly than those of the untreated controls. This accelerated movement of radiophosphorus from the femurs of treated rats could have resulted from an earlier action of parathyroid extract on the bones, or, as seems more likely, from the sustained action of parathyroid extract on phosphorus metabolism in the liver and the kidneys.

Calculations based on the values given in Table II show the average specific content of radiophosphorus in the kidneys of rats receiving parathyroid extract to be 18.5 per cent higher than that of the untreated controls during the first 24 hour interval. If insignificant variations from the control values are excluded (such as those shown by Rats E-1 and E-5, Table I), the above percentage is increased appreciably. Whether the higher activities observed for the kidneys of treated rats are due to the concentration of labeled phosphate in the process of being excreted, or result from a direct action of parathyroid extract on phosphorus metabolism in the kidneys, cannot be determined from the present data.

Although the per cent of the administered radiophosphorus excreted by the urinary and the fecal routes varied greatly for both the experimental animals and their controls, the excretion values usually indicate an increased urinary and a decreased fecal excretion of radiophosphorus by the hormone-treated animal.

SUMMARY

The percentage of labeled phosphate has been determined in the tissues and excreta of rats treated with parathyroid extract and untreated rats from 1 to 48 hours after the intraperitoneal injection of a single dose of Na_2HPO_4, containing radioactive phosphorus.

1. The accumulation of labeled phosphate reached higher values in the liver and kidneys and lower values in the femurs of rats receiving parathyroid extract than in their controls.
2. Throughout the 48 hour experimental period, a higher specific content of radiophosphorus was maintained in the liver and kidneys of the rats receiving parathyroid extract than in their controls.
3. During the last half of the first 24 hour interval, the specific content of radiophosphorus diminished more rapidly in the femurs of the rats given parathyroid extract than in the femurs of their controls.
4. Parathyroid extract appeared to produce an immediate increase in the urinary excretion of radiophosphorus, and a decrease in the fecal excretion of radiophosphorus that was first discernible 18 to 24 hours after the administration of the labeled phosphate.

The authors are greatly indebted to Professor E. O. Lawrence, director, and to Dr. Joseph G. Hamilton, and the rest of the staff of the Radiation Laboratory of the University of California for supplying the radiophosphorus used in these experiments. This work was also aided in part by Grant 535 from the American Medical Association, and by a gift of parathyroid extract from Eli Lilly and Company.

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SEPARATION OF OXYTOCIC AND PRESSOR PRINCIPLES OF POSTERIOR PITUITARY EXTRACTS*

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(Received for publication, April 13, 1944)

In 1928 Kamm, Aldrich, Grote, Rowe, and Bugbee (1) reported the separation of extracts of the posterior lobe of the pituitary gland into two fractions, respectively rich in oxytocic and in pressor activity, by fractional precipitation from 98 per cent acetic acid solution by acetone and ether and petroleum ether. An analogous process by Stehle and Fraser (2) involved fractional precipitation from mixtures of alcohol and ethyl acetate. Irving, Dyer, and du Vigneaud (3) likewise effected a considerable separation by electrophoresis. These reports confirmed the results of earlier investigations, particularly those of Dudley (4), who had concluded that the two principles differed in chemical and physical properties as well as in pharmacological action.

The preparation of two separate chemical fractions possessing the pharmacodynamic activities of the posterior pituitary has never been universally accepted as satisfactory evidence for the production of two substances by the gland. Certain investigators, notably Abel (5), held that the procedures used in separation of the principles were sufficient to cleave a hypothetical large molecule into smaller fragments. Rosenfeld (6) found that when press-juice from posterior lobes was subjected to ultracentrifugation fractions containing both pressor and oxytocic activity were sedimented at the same rate, in contrast to the products of Kamm which did not appreciably sediment under similar conditions. On the other hand, Irving and du Vigneaud (7) showed that the pressor component in fresh pressed juice from bovine pituitaries migrated more rapidly in electrophoretic experiments than the oxytocic principle. The best evidence for a single active component is given by van Dyke, Chow, Greep, and Rothen (8) who have described a homogeneous protein obtained from beef pituitary which contained the pressor, oxytocic, and antidiuretic hormones in constant ratio.

The present work was undertaken to provide a satisfactory procedure for

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* A preliminary report of this work was made before the Thirty-fifth annual meeting of the American Society of Biological Chemists (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **140**, p. ciii (1941)).

This investigation was financed by grants from Armour and Company, the Committee for Research in Endocrinology of the National Research Council, and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

obtaining the separated principles for chemical investigation. Of the various adsorbents reported (9–11) to remove posterior lobe hormones from solution, we found that zeolites showed a preferential adsorption of the pressor hormone. Accordingly, experiments were directed toward an efficient chromatographic separation with an adsorbent of this class. A readily available artificial zeolite is Decalso which, when ground to 70 mesh, is marketed as "permutit according to Folin." Solutions of posterior lobe extract are readily separated upon this zeolite. The procedure has the marked advantage that it can be applied to simple aqueous extracts of defatted pituitary powder and separation of the two activities can be achieved in the first stage of purification.

EXPERIMENTAL

Biological Assay—Oxytocic assays were made by means of the fowl blood pressure method of Coon (12). The vasopressor assays were conducted on cats anesthetized with phenobarbital. The U. S. P. reference standard was employed throughout. The accuracy of the pressor assay under favorable conditions is ±20 per cent; assay by the fowl blood pressure method for oxytocic activity is somewhat more accurate.

Preparation of Extracts and Separation by Adsorption on Zeolite

0.25 Per Cent Acetic Acid Extracts—The adsorbent in each case was 10 gm. of "permutit according to Folin," packed into a column (1.2 cm. inside diameter) while in suspension in 0.25 per cent acetic acid. The final column dimensions were 1.2 × 17 cm. The rate of flow by gravity alone was sufficient. The extract was made by triturating 15 gm. of acetone-desiccated posterior lobe powder with 150 ml. of 0.25 per cent acetic acid, heating to boiling, and filtering on a Buchner funnel with the help of 9 gm. of Hyflo Super-Cel. The filter cake was reextracted with another 100 ml. of 0.25 per cent acetic acid, and the two extracts were combined.

For each experiment either 40 or 50 ml. of the above solution were poured through the permutit column, and the filtrate was collected as Fraction 1. This was followed by two 100 ml. portions of 0.25 per cent acetic acid, collected as Fractions 2 and 3 respectively. Finally the remaining pressor hormone was eluted by 100 ml. of 5 per cent NaCl in 0.25 per cent acetic acid. This is Fraction 4. Three typical experiments are recorded in Table I.

Aqueous Extracts—The following experiments were analogous to the preceding ones, with the important difference that the extraction of the pituitary powder and the elution of the column were made with water instead of 0.25 per cent acetic acid as the solvent. To prepare the initial solution, 4.0 gm. of posterior lobe powder were triturated with 100 ml. of redistilled water and the insoluble material centrifuged out at high speed.

TABLE I

Separation of Oxytocic and Pressor Fractions from Acetic Acid Extracts of Pituitary Powder

		Experiment 134	Experiment 135	Experiment 147
		units	*units*	*units*
Before adsorption	Oxytocic	920	880	1150
	Pressor	880	800	1100
Fraction 1	Oxytocic	160	160	240
	Pressor	80	40	91
“ 2	Oxytocic	400	332	320
	Pressor	80	60	150
“ 3	Oxytocic	200	166	174
	Pressor	100	100	34
“ 4	Oxytocic	250	250	220
	Pressor	450	600	750
Total activity recovered	Oxytocic	1010	908	954
	Pressor	710	800	1025

TABLE II

Separation of Oxytocic and Pressor Fractions from Aqueous Extracts of Pituitary Powder

		Experiment 194	Experiment 195	Experiment 196
		units	*units*	*units*
Before adsorption	Oxytocic	1750	1750	1750
	Pressor	1600	1600	1600
Fraction 1	Oxytocic	1500	1300	1350
	Pressor	230	230	150
“ 2	Oxytocic	500	500	450
	Pressor	100	150	84
“ 3	Oxytocic	26		
	Pressor			40
“ 4	Oxytocic	160	220	200
	Pressor	1000	1000	1000
“ 5	Oxytocic		26	20
	Pressor	100	72	80
Total activity recovered	Oxytocic	2186	2046	2020
	Pressor	1430	1452	1354

The residue was extracted twice more with 50 ml. of water, each time in a similar manner. In these experiments the extractions were made at room temperature and the solutions were not heated. The extracts were chromatographed as in the previous experiments; the pressor fractions were eluted with 10 per cent sodium chloride solution (Fraction 4) and an additional fraction was collected by washing with 10 per cent sodium chloride in 5 per cent acetic acid (Fraction 5). In each of the experiments, the starting solution was 50 ml. of the same extract. The results are recorded in Table II.

Removal of Pressor Activity by Repeated Adsorption

Two experiments were conducted with aqueous extracts similar to those recorded in Table II, but in addition the oxytocic fraction was poured through a second permutit column identical with the first, in order to ascertain how much additional pressor hormone could be removed by a second passage.

1.5 gm. of posterior lobe powder were extracted three times with redistilled water, by use of 30, 25, and 20 ml. portions, and the unextracted residue was removed by centrifuging at high speed. 50 ml. of this extract were used in each experiment. All columns were 1.2 × 17 cm.; 10 gm. of permutit were used. Since the starting extract was milky in appearance and remained so in the eluate, the water entrained in the column could be eliminated by collecting the first fraction when the milky liquid appeared. The entrained solvent, which amounted to 13 ml., was inactive and was discarded. Fraction 1 represents the 50 ml. of starting material.

TABLE III

Repeated Adsorption of Oxytocic Fractions

Fraction 1		Experiment 198	Ratio, oxytocic/pressor	Experiment 199	Ratio, oxytocic/pressor
		units		*units*	
After 1st adsorption	Oxytocic	2000	5.0	1880	6.7
	Pressor	400		280	
" 2nd "	Oxytocic	1600	8.0	1800	11.6
	Pressor	200		155	

The 50 ml. of initial extract contained 2000 units of oxytocic and 2000 units of pressor activity. In each case after removal of a small aliquot for assay, Fraction 1 was poured through a second fresh column and again the milky eluate only was collected. The results are recorded in Table III.

This last experiment was repeated with the same extract and the first eluate was twice again chromatographed, with 10 gm. of permutit for each adsorption. The results are recorded in Table IV.

Separation of Larger Quantities—105 gm. of acetone-desiccated posterior lobe powder were triturated with 3 liters of redistilled water and filtered with the aid of Hyflo Super-Cel. The residue was extracted with 2 more liters of water and the extracts combined. It may be noted that filtration is very slow and centrifugation is preferable.

A large column, 5 × 102 cm., of permutit was prepared with 1 kilo of adsorbent. The pituitary extract was allowed to pass through the column by gravity and was followed by a 3 liter portion of water. Both filtrates were combined as Fraction 1. The column was then washed with 4 liters of 10 per cent NaCl solution, designated as Fraction 2. Fraction 1 was poured through another column of permutit (1 kilo) and the column washed once with 3 liters of water. The combined filtrates were assayed as Fraction 3. Here, too, the turbidity of the extract indicated when the active material had started to come through, so that liquid initially present in the column was separated and discarded. The results of two experiments are recorded in Table V.

TABLE IV

Repeated Adsorption of Oxytocic Fractions

Experiment 200, Fraction 1.

	After one adsorption	Ratio, $\frac{\text{oxytocic}}{\text{pressor}}$	After two additional adsorptions	Ratio, $\frac{\text{oxytocic}}{\text{pressor}}$
	units		*units*	
Oxytocic . . .	1925	12.5	1750	20
Pressor	154		85	

Separation of Oxytocic and Pressor Activity from Fresh Glands—Since the ordinary methods for collection and desiccation of posterior lobes offer opportunities for secondary changes in a protein molecule, the following experiments were performed with the view of minimizing such alterations in the hormone or hormones. In Experiment 189 (Table VI), the pituitaries were obtained at the slaughter-house, at the time the glands are ordinarily removed from the head, and were immediately frozen on solid CO_2. The beef pituitary gland is ordinarily not removed from the skull until 1 hour or longer after death, since the rest of the carcass must pass inspection before the head is opened. This seemed to offer possibility for postmortem change and therefore, with the cooperation of Armour and Company and a representative of the Bureau of Animal Industry, we collected some twenty-five glands which were removed and frozen on CO_2 within 10 minutes after the death of the animal. The results with these glands are given in Experiment 191. The pituitaries were dissected while frozen and dehydrated in a high vacuum. Toward the end of the dehydration the flask containing the glands was chilled in a salt-ice mixture, so

TABLE V

Large Scale Separation of Pressor and Oxytocic Fractions from Aqueous Extracts of Pituitary Powder

		Experiment 207	Experiment 212
		units	*units*
Before adsorption	Oxytocic	147,000	160,000
	Pressor	138,000	130,000
Fraction 1	Oxytocic	171,000	140,000
	Pressor	16,000 (Approximate)	5,000 (Approximate)
" 2	Oxytocic	18,400	
	Pressor	140,000	120,000
" 3 (2nd adsorption of Fraction 1)	Oxytocic	168,000	192,000 (?)
	Pressor	*	*

* The responses in the pressor assay of these samples are completely atypical. Instead of the normal rise from a base-line these high oxytocic samples invariably show a preliminary drop followed by a return to normal or beyond. This secondary rise appears to be compensatory for the drop and ordinarily its magnitude appears proportional to the size of the previous fall. For this reason, assays for pressor activity in samples such as Fraction 3 are completely unreliable.

TABLE VI

Separation of Pressor and Oxytocic Fractions from Glands Subjected to Minimal Denaturation and Autolysis

		Experiment 189*	Experiment 191†
Extract, *ml*		5 7	7 5
Powder extracted, *mg.*		85	94.5
		units	*units*
Fraction 1	Oxytocic	88	72
	Pressor	8	5
" 2 (10 ml. water)	Oxytocic	3	5
	Pressor	2	1
" 3 (10 " ")	Oxytocic	3	10
	Pressor	1.2	1
" 4 (10 " 0.25% acetic acid)	Oxytocic	8	10
	Pressor	3	2
Fraction 5 (10 " 10% NaCl)	Oxytocic	7	15
	Pressor	62	58

* Glands removed from head approximately 1 hour after slaughter.

† Glands removed from head approximately 10 minutes after slaughter.

that at no time, until all water had been removed, were the glands exposed to a temperature higher than 0°. The dried posterior lobes were extracted with 30–60° petroleum ether, powdered, sifted, and dried in a high vacuum before extraction. Table VI records the results of two typical experiments in which these powders were extracted with redistilled water as before and poured over 10 gm. of permutit.

DISCUSSION

These experiments present a rapid, simple, and convenient method for separation in high yield of the oxytocic and pressor pituitary principles by an adsorption process. As the adsorbent used is capable of exchanging cations with the solution with which it is in contact, the pressor hormone, which has been shown to be a more basic ampholyte (7) than the oxytocic principle, is adsorbed by this "base exchange" substance. The more basic character of the pressor hormone is consistent with the higher arginine content of this fraction (13). The degree of purification effected in this procedure is not great; estimations of solids were not made because of the high salt content of extracts after passage through permutit. The separated principles, however, offer a marked advantage for the further purification of the two products, since the losses are slight and the separation of the two activities is fairly complete.

In these experiments, a careful effort was made to avoid any alteration of the hormone molecules other than exposure to the surface of the zeolite. As the effect of this agent on a protein molecule such as van Dyke, Chow, Greep, and Rothen (8) have described has not been investigated, it is unknown whether permutit effects a separation of the biological activities from the protein.

The protein isolated by van Dyke *et al.* (8) represents about 5 per cent of the total biological activity which may be extracted from the gland by efficient methods. Of the remaining 95 per cent, a significant fraction is dialyzable. We have made similar observations on aqueous extracts of the gland before and after adsorption on permutit.[1] A significant proportion of the activity of the glandular extracts is evidently associated with relatively small molecules. The activities of the separated principles are qualitatively identical with those of the protein molecule from which they are cleaved, judged by the biological assay procedures which have been applied to the products. That they are not quantitatively identical is indicated by the following calculation: van Dyke *et al.* have stated that 0.061 mg. of protein is the equivalent of 1 oxytocic unit. Potts and Gallagher (13) have calculated a minimum molecular weight of approximately 1300 for the oxytocic fraction from the cystine and tyrosine content of highly purified preparations, in which 0.0014 mg. was the equivalent of 1 U. S. P. unit. From the molecular weight of the protein (30 000) it can be calculated that 4.3 per cent is oxytocic hormone, assuming a minimum molecular weight of 1300 for this substance. Accordingly the oxytocic unit of the separated principle (if this were obtained unaltered in activity) should be 0.061 mg. × 0.043 = 0.0026 mg. of solids. Since the preparations studied by Potts and Gallagher were almost twice as potent as this, it is obvious that either the protein was impure or the biological activity is quantitatively different in the protein and in the separated principle.

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[1] Unpublished experiments with Dr. John A. Vaichulis.

The procedure by which the two activities of the posterior pituitary can be separated is so simple, and so high a percentage of the total activity can be extracted, that the conclusion that a single protein with multiple biological activity is the hormone of the posterior pituitary seems unwarranted.

SUMMARY

A simple and convenient method for the separation of the oxytocic and pressor principles from aqueous extracts of desiccated posterior lobes has been described. The pressor principle is adsorbed on "permutit according to Folin," whereas the oxytocic principle is apparently not adsorbed. Elution of the pressor substance was accomplished with sodium chloride solution. High yields of the separated principles were obtained.

The authors wish to express their appreciation to Miss Lillian Hunter who performed the biological assays; to Professor E. M. K. Geiling who provided space for the assays and helped us with the dissection of the fresh glands; and to Professor E. A. Evans, Jr., who made many suggestions in the preparation of the manuscript.

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MICRODETERMINATION OF VOLATILE FATTY ACIDS IN BLOOD

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(Received for publication, April 5, 1944)

Volatile fatty acids (VFA) may be separated by fractional distillation, or a mixture of two or three determined by mathematical analysis of the distillation curve. Duclaux (1) distilled directly, Dyer (2) distilled with steam, and Olmsted, Whitaker, and Duden (3) from a saturated solution of magnesium sulfate. Such methods require higher concentrations of VFA than are present in protein-free filtrates of blood from the normal general circulation. If the distillate is to be made alkaline, evaporated, and redistilled to obtain the distillation curve, larger quantities of blood than are usually available are required. The following method is usually limited to total VFA (4). Only in blood following intravenous injections, portal blood during absorption, or in concentrates of large volumes of blood distillate have individual acids been determined.

Procedure

Blood is drawn in a graduated syringe and spurted into 5 times its volume of 2 per cent $ZnSO_4$-$7H_2O$ and mixed. (At this point it may be refrigerated if the determination cannot be made immediately, but changes do take place and samples showing the brown color of methemoglobin give abnormal values.) 12 cc. are mixed with 2 cc. of half normal NaOH and centrifuged or filtered. 7 cc. of this Somogyi (5) filtrate are considered the equivalent of 1 cc. of blood and are introduced into the still at *A*, Fig. 1, by means of a 7 cc. pipette. 0.5 cc. of syrupy phosphoric acid (that has had steam blown through it while hot) is added at *A*. Cooling water is circulated through the condenser and a 50 cc. Erlenmyer flask with a mark at 30 cc. is placed under *C*. Micro burners are lighted under the glycerol bath and steam generator, and when the bath reaches 120° the stopper is inserted in *A*, causing steam entering *B* through a rubber tube to bubble through the filtrate in the still. The burners are regulated so that the bath remains at 120° and the water seal opposite *E* is not broken while 30 cc. of distillate are collected. 3 drops of 0.04 per cent brom-thymol blue are added to the distillate and air that has passed through sodium hydroxide solution and wet permutit is bubbled in a fine stream through it for 10 minutes and during the titration. It is titrated with 0.01 N CO_2-free

NaOH in a Lochte and Hoover (6) burette graduated in thousandths of a cc. to the color of a standard of 30 cc. of CO_2-free water, 3 drops of indicator, and 3 mg. of sodium acetate. The end-point is viewed in front of a daylight fluorescent lamp with a flashed opal glass diffuser.

A blank determination is made by substituting Ringer's fluid for an equal quantity of blood. The titer of this blank is subtracted from the titer of blood.

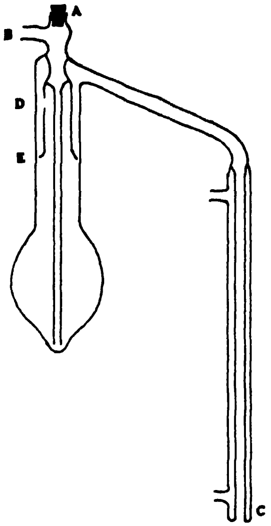

Fig. 1. Micro still of 50 cc. capacity. *A* stoppered intake, *B* inlet for steam, *C* outlet for distillate, *D* hole in trap, *E* annular (0.25 mm) space at lower margin of trap to be closed by water seal. The space may be smaller and does not have to be uniform, but must not be larger.

The still is emptied by suction at *B* while the stopper remains in *A*, and is rinsed by suction while a small beaker of distilled water is held under *C*. A battery of four stills can be run at the same time.

DISCUSSION

The chief advantages in the still shown in Fig. 1 are its small size (50 cc.), absence of ground joints, and very efficient trap. The trap is an inverted test-tube in the neck of the distilling flask with a hole opposite *D* through which any spray is projected onto the wall of the flask while the gap opposite *E* is closed by a water seal. The glass-blower is instructed to test the water seal to see that it will hold against gravity. Since the force of the steam is opposed to gravity, the seal will stand the pressure due to a fair rate of distillation. The temperature, 120°, of the bath was found to be correct for maintaining the volume in the still at 7.5 cc. Therefore the temperature in the still is only slightly above 100° and the insertion of a thermometer within the still itself is unnecessary. A mixture of glycerol (or permanent antifreeze) and water that boils at 120° may be maintained by adding water to a mark on the bath as it boils away, or the temperature may be maintained by regulating the burner.

Performance of Still with Known Fatty Acids—Since blood is diluted 7 times in deproteinizing, 1 cc. of acid of the recorded strength was introduced into the still with 6 cc. of water. The distillation rate of acid is lowered by this dilution. On the other hand, if the blood filtrate were concentrated to the volume of the original blood before distillation, HCl would distil and interfere with the titration.

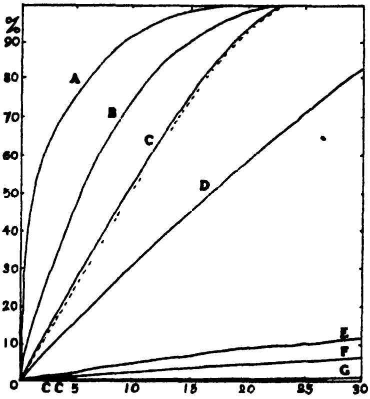

FIG. 2. Distillation rates, expressed as cc. of distillate on the abscissa and per cent of fatty acid distilled on the ordinate, when the still is charged with 6 cc. of water, 0.5 cc. of phosphoric acid, and 1 cc. of one of the following acids: (Curve A) 0.02 N caproic; (Curve B) 0.02 N butyric; (Curve C) 0.02 N acetic (dash line, 7 cc. of blood filtrate representing 1 cc. of VFA-free blood and 0.02 cc. of 1 N acetic acid); (Curve D) 0.02 N formic; (Curve E) 0.1 N pyruvic; (Curve F) 0.02 N β-hydroxybutyric; (Curve G) 0.1 N lactic.

Fig. 2 shows the distillation curves of a number of VFA made by titrating each 5 cc. portion of the distillate. The dash line represents 0.02 cc. of normal acetic acid added to each cc. of VFA-free blood, followed by the usual procedure except for the separate titration of each 5 cc. portion of the distillate. The close parallel to the solid line for acetic acid indicates that it is not appreciably adsorbed by the zinc hydroxide. Fig. 2 shows that 0.02 N caproic acid is all distilled in 20 cc., 0.02 N butyric or acetic acid in 25 cc., whereas only 85 per cent of 0.02 N formic acid is distilled in 30 cc.

Only 12 per cent of 0.1 N pyruvic acid, 7 per cent of 0.02 N β-hydroxybutyric acid, and 1 per cent of 0.1 N lactic acid are distilled in 30 cc. The distillation rate depends on the ratio of the non-polar (CH_2) groups to the polar (COOH, CO, and COH) groups.

The concentrations in the still that gave distillates of 30 cc. of zero titration were 0.00002 N caproic, butyric, or acetic acid, 0.0002 N pyruvic acid, 0.0006 N β-hydroxybutyric acid, and 0.007 N lactic acid.

In order to obtain an unmistakable qualitative test for acetic acid with lanthanum nitrate, iodine, and ammonia (Feigl (7)) it was necessary to distil the protein-free filtrate of a pint of blood, make it alkaline, evaporate to the volume of 2 drops, and distil 1 drop in a 5 cc. micro-Kjeldahl still with the receiver cooled in a freezing mixture.

Although not enough hydrochloric acid distils from blood filtrates to give a qualitative test with silver nitrate, theoretically some should come over; hence the blank was made by treating Ringer's fluid in the same way as blood.

The VFA of normal human or dog venous blood is 0.0003 N. In certain experiments in which acetate was injected into the vein or introduced into the alimentary canal the value rose to about 0.003 N and it is probable that 90 per cent of this was acetic acid.

SUMMARY

1. A rapid method for the determination of volatile fatty acid in the filtrate of 1 cc. of blood is described.

2. Distillation curves for different acids as they would distil from blood filtrates are figured.

3. The titer of volatile fatty acids of normal blood from the general circulation of man or dog (after subtraction of the blank) is 0.0003 N.

Thanks are due to K. A. C. Elliott for suggestions, Thomas M. Walton of the Precision Thermometer and Instrument Company, Philadelphia, for stills that reduce breakage, Dr. Harry M. Eberhard for blood samples, and R. H. Hamilton for pure pyruvic acid.

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COMPARISON OF CRUDE AND PURIFIED PREPARATIONS OF A LEUCYLPEPTIDASE ASSOCIATED WITH BEEF MUSCLE*

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(Received for publication, March 20, 1944)

Berger and Johnson (1) have established the existence of a peptidase which is activated by magnesium and manganese salts and is particularly adapted to splitting initial leucine residues from peptides. The enzyme has been found in hog erepsin, and in several species of plants and bacteria. It is thus of wide-spread occurrence. A similar enzyme has now been found in association with beef muscle. Some of the properties of a purified preparation of this peptidase have been studied and compared with those of a crude glycerol extract of beef muscle. As a result of this comparison it appears that more than one peptidase occurs in muscle. The crude extract is capable of hydrolyzing peptides other than leucyl peptides, whereas the purified enzyme is not.

Methods

Determination of Activity—3 ml. of enzyme solution plus 1 ml. of water or heavy metal salt solution (as an activator) were added to 65.8 mg. of *dl*-leucylglycine dissolved in 3 ml. of 0.1 M ammonia-ammonium chloride buffer. This was equivalent to 0.05 mM of peptide per ml. of digestion mixture. At various times during the digestion at 40°, 1 ml. was removed and titrated with 0.01 N NaOH by a formol method (2). In this case an increased titration of 5 ml. would be equivalent to 100 per cent splitting of the (racemic) peptide. 1 activity unit, [Pep. u.], has been defined as the quantity of enzyme which will cause 5 per cent total hydrolysis of the *dl*-leucylglycine in 1 hour at 40°.

Preparation of Purified Enzyme—200 gm. of ground lean meat, previously frozen, were stirred into 400 ml. of a solution prepared by mixing 1 volume of glycerol with 1 volume of water. The pasty mass was filtered by gravity overnight at 1°. The extract was stored at −17.8°.

It is referred to as the crude extract. To 300 ml. of this extract, 110 ml. of cold acetone were added. The mixture was centrifuged at −10°.

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* Enzyme Research Laboratory Contribution No. 91. This work was done on Bankhead-Jones funds. Some of the results are from a thesis submitted to Georgetown University in 1941 by Sigmund Schwimmer in partial fulfilment of the requirements for the degree of Master of Science.

Another 110 ml. portion of acetone was then added to the supernatant liquid. The precipitate resulting from the second addition of acetone was centrifuged out, and then dissolved in 150 ml. of 0.1 N ammonia-ammonium chloride buffer, pH 8.0. Ammonium sulfate was added to the solution to the point of 0.65 saturation and the precipitate so produced was removed by centrifuging and dissolved in 20 ml. of water. To this solution were added 6 ml. of a solution saturated with respect to ammonium sulfate and 0.015 N with respect to acetic acid. The precipitate so formed was discarded. The supernatant was made 0.5 saturated with ammonium sulfate, resulting in a precipitate of protein very rich in enzyme. This precipitate was dissolved in 10 ml. of 30 per cent glycerol. The purification achieved by this procedure (Table I) resulted in a 24-fold increase in activity, in units per mg. of protein nitrogen. About two-thirds of the activity of the original glycerol extract was lost during purification.

TABLE I

Purification of Beef Leucylpeptidase

Preparation	Specific activity		Recovery of enzyme
	$\left[\text{Pep. u.}\right]\frac{\text{L. G.}}{\text{mg P. N}}$		$\left[\text{Pep. u.}\right]\frac{\text{L. G.}}{\text{mg. P. N}}$
	No activator	0 001 M $MnCl_2$	0.001 M $MnCl_2$
Crude glycerol extract	1.00	2.4	700
Protein in ammonia-ammonium chloride solution		15	300
Final preparation	22	57	200

Results

Activation by Manganese, Magnesium, and Copper—Manganese salts were most effective in increasing the activity of the crude glycerol extract on leucylglycine. Magnesium and copper were also effective, while iron had little effect (Table II). Cyanide and cysteine did not materially affect the results. The activation of the purified leucylpeptidase by Mn was found (Table I) to be the same; *i.e.*, activation resulted in approximately a 2.5-fold increase in both cases.

Course and Extent of Hydrolysis—Fig. 1 shows that the enzyme hydrolyzes *dl*-leucylglycine to a maximum of 50 per cent. The courses of hydrolysis of this substrate by the crude and purified preparations are about the same. The shape of the curve is typical of enzyme reactions when the substrate is not very dilute; the velocity is independent of the substrate concentration until the latter is sufficiently small to result in a typical "die away" curve. That is, the initial concentration of substrate is apparently much higher than the K_m value (the dissociation constant of Michaelis and Menten) for this particular enzyme. The use of high substrate concentrations seems to be a common practice in peptidase studies (3, 4).

TABLE II

Effect of Salts on Activity of Crude Extract

Salt added	Concentration			
	10^{-2} M	10^{-3} M	10^{-4} M	No salt added
	Per cent hydrolysis (0.5 ml. extract, 1 hr.)*			
Control				12.5
$MnCl_2$	12	27	22†	
$FeSO_4$	10	12†	13	
$MgCl_2$	19	15	14	
$CuSO_4$	18	20		
NaCN	10	13		

* *dl*-Leucylglycine used as substrate.

† Prior addition of cysteine (0.005 M) gave a corresponding value of 20.5 for $MnCl_2$ and 13.5 for $FeSO_4$.

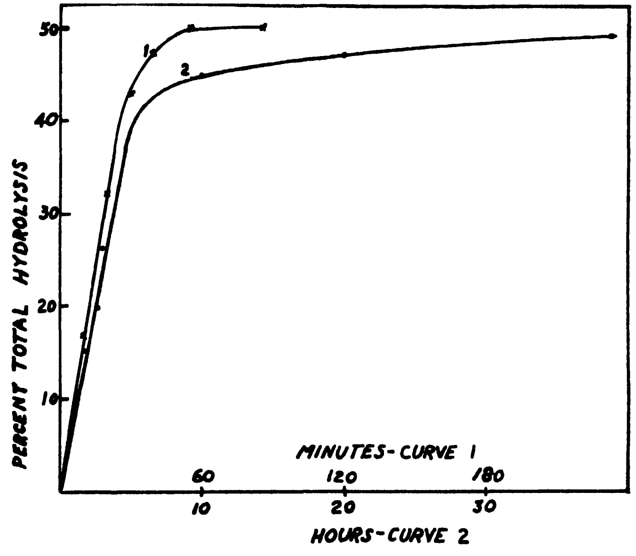

FIG. 1. Course and extent of hydrolysis by purified enzyme (Curve 1) and crude extract. *dl*-Leucylglycine was used as substrate in the presence of 0.001 M $MnCl_2$; 0.40 mg. of purified enzyme and 0.50 ml. of crude extract were used.

The kinetics of the purified leucylpeptidase and also the extent of activation (about 2.5-fold) obtained with manganese are essentially the same as were observed with the crude extract. The enzyme appears to have undergone little if any change. Purification has, however, removed

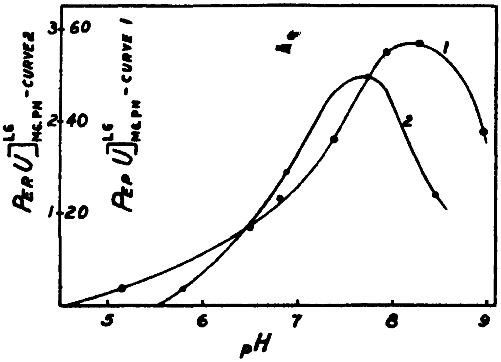

FIG. 2. pH optima of purified enzyme (Curve 1) and of crude extract (Curve 2), in the presence of 0.001 M added Mn.

TABLE III

Specificity of Purified and Crude Enzyme

Substrate 0 05 mM per ml	Per cent hydrolysis (2 hrs.)			pH
	Purified enzyme		Crude glycerol extract	
	0.027 mg.	pH	0 1 ml	
dl-Leucylglycine	34.1	7.95	9	7.8
"	24	7.1		
dl-Leucyldiglycine	25.1	6.9	50*	7.5
Glycyl-*l*-leucine	0.8	7.1	7*	6.8
Glycylglycine	0.2	4.0	20*	6.7
"	0.0	7.5		
Glycyl-*l*-alanine	0.0	7.5		
Glycine anhydride	0.2	7.0		
dl-Alanylglycine	0.0	6.8	50*	7.0
dl-Alanyl-*dl*-alanine	0.0	7.5		
Chloroacetyl-*o*-nitraniline	0.0	7.2		
m-Nitrobenzoylglycine	−0.3	7.0		
Cathepsin hemoglobin†	0.0	3.7	+	3.7
Trypsin hemoglobin†	0.0	7.4		

* Hydrolysis after 20 hours.

† Determined according to Anson (5).

completely the power to split alanyl or glycyl peptides. The inference seems allowable that another peptidase has been removed, rather than a substance that causes leucylpeptidase to digest other peptides as well.

pH Optima—The pH optima of the two preparations assayed in the presence of 0.001 M $MnCl_2$ are quite different (Fig. 2). The optimum for the purified enzyme at pH 8.3 is additional indication that the enzyme is similar to that previously described (3).

Specificity—The purification has resulted in a highly specific, as well as active preparation. Table III shows the action of the two preparations on various substrates. Of the peptides tested only *dl*-leucylglycine and *dl*-leucyldiglycine were hydrolyzed by the purified enzyme. In agreement with Johnson *et al.* (3), the rate of splitting of leucylglycine is equal to that of leucyldiglycine at the same pH. The maximum hydrolysis of leucyldiglycine corresponded to the splitting of one bond per molecule.

TABLE IV

Stability of Purified Enzyme toward Dialysis

Condition of experiment	$[\text{Pep. u.}]^{\text{L. G.}}_{\text{mg. P.N}}$ (pH 7.7)	
	No activator	0.001 M $MnCl_2$
No dialysis of glycerol solution	22	50
0.75 ml. glycerol solution + 0.75 ml. H_2O dialyzed against distilled H_2O at 0° for 3 days	12	25
0.75 ml. glycerol solution + 0.75 ml. 0.005 M $MnCl_2$ dialyzed against 0.001 M $MnCl_2$ for 3 days at 0°	2	17

TABLE V

Survival of Crude Enzyme As Function of pH at 0°

pH	Per cent activity remaining after*	
	2 hrs	19 hrs.
2.95	3	0.5
3.75	95	21
5.90	100	98
8.70	96	94
10.00	97	80

* *dl*-Leucylglycine used as substrate in presence of 0.001 M $MnCl_2$.

Stability—The purified enzyme proved to be rather unstable. Storage of an aqueous solution at 0° for 2 weeks resulted in a 75 per cent loss in activity. Glycerol stabilized the enzyme. The stabilizing effect of glycerol and the instability of peptidases in dilute aqueous solution are in agreement with previous observations (6). Paralleling the results of Smith and Bergmann (7) on the stability of leucylpeptidase in hog erepsin, the purified enzyme was shown to be less stable in the presence of manganese than in water (Table IV). The amount of manganese present during this dialysis was roughly a tenth of the quantity required for maximum activation; hence the increase in activity observed on the addition of more manganese to the dialyzed material.

The survival of the crude enzyme at various pH values after 2 and 19 hours is shown in Table V.

SUMMARY

A highly active specific enzyme capable of hydrolyzing leucylglycine and leucyldiglycine, but not simple glycine or alanine peptides, has been found associated with beef tissue. Its properties have been compared with a crude glycerol extract of beef muscle. The properties investigated indicate that the purified enzyme is a leucylpeptidase, whereas the starting material contained more than one peptide-splitting enzyme.

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INFLUENCE OF SOME SULFUR-CONTAINING COMPOUNDS ON LIVER LIPID CONTENT OF YOUNG WHITE RATS

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(Received for publication, April 21, 1944)

Recent studies in lipid metabolism have shown that a relationship exists between the content of sulfur-containing amino acids of the diet and the percentage of "fat" in the liver. Large amounts of lipids (20 to 30 per cent) are deposited in this organ when a ration high in fat, low in protein, and deficient in choline or its precursors is fed to the young white rat for a comparatively short time (2 weeks). This accumulation of unusual amounts of "fat" in the liver can be prevented by merely supplementing such a diet with 0.5 per cent of methionine. On the other hand a mobilization of additional amounts of lipids into this organ is observed when 0.5 per cent of cystine is added to the ration. It is generally believed that methionine exerts this so called lipotropic action by transmethylation, whereby its labile methyl group is used for the synthesis of choline. Numerous methylated compounds have been studied with regard to lipotropic effect (*cf.* Moyer and du Vigneaud (1) for a summary of the literature). It is clear from their summation that not all methylated products are lipotropic but no good reason has been presented to explain the inactivity of some and the activity of others. Different explanations have been given to account for the deposition of additional amounts of "fat" in the liver when cystine is added to the above ration. According to Griffith (2) the additon of cystine to such a diet may improve the nutritional state of the animal to such an extent that the choline requirements are increased. In contrast to this it has been suggested that this amino acid exerts a direct toxic effect on the animal. Smythe (3) believes that the action of the acid may be attributed to the liberation of hydrogen sulfide by an enzyme occurring in the liver. It was the purpose of this investigation to obtain additional data relating to these opposing effects of methionine and cystine.

The method of attack consisted in administering (intraperitoneally in most cases) substances containing sulfur to young male rats on a diet which will produce fatty livers. The experiments were usually terminated at the end of approximately 2 weeks and the liver lipid content was determined in the manner previously described (4). In most of the experiments, the system of paired feeding of litter mates was employed. The basal diet contained 3 per cent Celluration (Fisher), 15 per cent casein (Labco), 37 per cent glucose, 40 per cent lard, and 5 per cent salt mixture (5). In addition, each rat received 1 dry yeast tablet (500 mg.) and 2 drops of cod liver oil daily. All organic compounds were analyzed for sulfur by the Parr bomb method and were found to be of satisfactory purity.

Influence of Inorganic Sulfide on Liver Lipid Content—In the light of Smythe's idea that the toxicity of cystine may be due to the liberation of hydrogen sulfide (3), it seemed desirable to determine the effect of inorganic sulfide on liver lipid content. The product employed was commercial $Na_2S \cdot 9H_2O$ which was shown to be 98 per cent pure by iodometric analysis. As was expected, the product was quite toxic, but it was possible to administer 5.5 mg. (as anhydrous Na_2S) per kilo intraperitoneally. The experimental rats received daily intraperitoneal injections of a solution prepared by adjusting the pH of an aqueous solution of the sulfide to 9 by the addition of dilute HCl. Table I shows the amounts administered to the individual rats. The control animals were given intraperitoneal injections of water equal in volume to that used in the administration of the sulfide to the experimental animals. In Series A and B the diet was fed *ad libitum;* in Series C paired feeding was employed. Table I shows that in Series A the average liver lipid content of the control animals (24.9 per cent) is higher than the corresponding value (19.6 per cent) for the rats receiving the sulfide. In the second series (B) the average figures for the control and experimental animals are 18.0 and 20.3, respectively, and in Series C (with the exception of Pair II) the value for the liver lipid content of each experimental animal was smaller than that of its paired control. No evidence is thus presented in support of the hypothesis that the effect of cystine on the content of liver lipids is due to the liberation of hydrogen sulfide.

The data on food intake are included in Table I because of a suggestion of better growth in the case of the rats, in Series B and C, obtaining the sulfide. In the former the average gain in weight for the 19 day period was 40.9 gm. for the experimental group and 37.8 for the controls. In Series C, which is better controlled, every rat receiving the sulfide gained more weight than its paired control. These data suggest that additional tissues might have been laid down by the rats receiving the supplement. A definite decision regarding this is withheld, since such factors as activity, water intake, and urine secretion were not controlled.

Influence of Other Types of Sulfur Compounds on Liver Lipid Content—In these experiments various types of sulfur compounds, a sulfone (methionine sulfone), a sulfide (dimethyl sulfide), a disulfide (dimethyl disulfide), a thion (S-methylisothiourea), the ester of a thion-thiol acid (methylxanthogenate), and a sulfonium compound (trimethylsulfonium chloride), were investigated. Unless otherwise stated paired feeding of young male litter mates was employed.

TABLE I

Influence of Inorganic Sulfide on Liver Lipid Content

The duration of the experiment was 19 days in Series A and B, and 22 days in Series C. The amount of supplement indicated represented the quantity (calculated as Na_2S) administered intraperitoneally during the entire period. The small letters and roman numerals in Series C refer respectively to the litter and the pair used in paired feeding. Liver lipids are calculated on the fresh basis.

Series	Rat No	Supplement (Na_2S) added	Initial weight	Gain in weight	Food intake	Liver lipids
		mg	*gm.*	*gm*	*gm*	*per cent*
A	1	23	117	33	132	15.9
	2	23	125	27	141	19.5
	3	23	117	21	114	20.7
	4	17	130	33	127	25.6
	5	17	109	34	109	16.1
	6	17	122	31	145	20.3
	7	0	116	21	119	25.4
	8	0	129	36	130	28.9
	9	0	131	26	134	20.3
B	10	32	122	23	189	22.8
	11	32	168	54	203	16.2
	12	32	147	41	194	25.3
	13	32	145	34	171	23.8
	14	32	155	53	194	20.4
	15	32	152	40	193	13.1
	16	0	143	43	196	18.4
	17	0	142	31	195	17.3
	18	0	138	40	190	21.8
	19	0	149	42	185	17.0
C	I-1-a	31	119	28	139	18.6
	I-2-a	0	106	18	137	26.9
	II-3-a	31	121	33	151	28.8
	II-4-a	0	111	29	151	27.3
	III-5-b	31	117	40	151	25.4
	III-6-b	0	120	38	152	26.0
	IV-7-b	31	128	40	162	23.9
	IV-8-b	0	118	31	157	25.1
	V-9-c	31	141	54	178	24.1
	V-10-c	0	134	46	177	28.9
	VI-11-c	31	130	52	144	30.2
	VI-12-c	0	120	42	142	33.9

In a former report from this laboratory it was announced (6) that methionine sulfoxide possessed lipotropic activity. This was anticipated in view of the report by Bennett (7) that this sulfoxide could be substituted in the

Table II

Influence of Methionine Sulfone and Dimethyl Disulfide on Liver Lipid Content

The duration of the experiment was 20 days in Series D and E, 8 days for Series F, and 15 days for Series G. The amount of supplement indicated represents the quantity administered during the entire period. The rats in Series F received the supplement subcutaneously. In the remaining cases it was given intraperitoneally. The letters and roman numerals in the second column refer respectively to the litter and the pair used in paired feeding. Liver lipids are calculated on the fresh basis.

Series	Rat No	Supplement added		Initial weight	Gain in weight	Food intake	Liver lipids
			mg.	*gm.*	*gm.*	*gm.*	*per cent*
D	I-1-a	Sulfone	0	159	42	183	25.8
	I-2-a	"	950	150	44	183	29.3
	II-3-a	"	0	113	1	122	20.5
	II-4-a	"	950	101	−9	122	23.8
	III-5-a	"	0	144	47	186	19.2
	III-6-a	"	950	151	42	183	29.7
	IV-7-b	"	0	155	51	190	26.7
	IV-8-b	"	950	154	46	189	28.0
	V-9-b	"	0	125	12	124	22.6
	V-10-b	"	950	103	5	123	23.9
	VI-11-b	"	0	140	35	166	34.5
	VI-12-b	"	950	132	31	159	22.9
E	35	Disulfide	0	143	43	196	18.4
	36	"	0	142	31	195	17.5
	37	"	0	142	28	194	15.3
	38	"	0	138	40	190	21.8
	39	"	0	149	42	185	17.0
	40	"	260	146	36	147	8.3
	41	"	260	155	49	191	14.6
	42	"	260	123	18	160	6.7
	43	"	260	135	22	167	12.8
F	I-46-c	"	0	120	26	80	13.2
	I-47-c	"	100	122	22	80	12.6
	II-48-c	"	0	124	12	74	12.1
	II-49-c	"	100	119	12	74	7.5
	III-50-d	"	0	123	20	80	15.0
	III-51-d	"	100	123	24	80	11.4
	IV-52-d	"	0	108	14	71	11.8
	IV-53-d	"	100	112	9	71	8.9
G	V-54-e	"	0	130	40	103	12.6
	V-55-e	"	130	124	28	104	13.0
	VI-56-e	"	0	150	51	114	23.4
	VI-57-e	"	130	136	39	114	18.5
	VII-58-e	"	0	151	39	129	28.3
	VII-59-e	"	130	144	38	129	22.4
	VIII-60-f	"	0	112	33	89	24.1
	VIII-61-f	"	130	95	5	87	20.3

diet of the white rat for the purposes of growth. Bennett subsequently observed (8) that the more highly oxidized derivative, methionine sulfone, was not effective in promoting growth. It seemed desirable therefore to determine the effect of this sulfone on liver lipid content. The product was synthesized according to the procedure of Toennies and Kolb (9). Table II gives the data on six pairs of rats, and clearly demonstrates that no lipotropic action is in evidence when the sulfur of methionine is oxidized to the sulfone, the average value for the rats receiving the methionine sulfone being 26.3 per cent as compared with the mean value of 24.9 for the controls.

Dimethyl disulfide was chosen for study, since it can arise from the decarboxylation of dithiodiglycolic acid, a compound reported to be lipotropic by Singal and Eckstein (6). This substance, synthesized according to the method of Blackburn and Challenger (10), proved to be quite toxic, the fatal dose (intraperitoneal) ranging from 90 to 100 mg. per kilo. Evidence of the lipotropic action of this disulfide is presented in Table II. Rats 35 to 43 inclusive received the diet *ad libitum* for 20 days. The liver lipid content of all of the experimental rats in this group (Series E) was significantly lower than that of the controls. This observation is confirmed by the paired feeding experiments with the other rats. In Series G the daily dose was decreased and the product administered dissolved in oil (Mazola); the controls received an equivalent amount of the oil alone by the same route. In Series F the dose was increased and was given subcutaneously twice daily. These animals became so irritable that it became necessary to terminate the experiment at the end of the 8th day.

In the experiments in which dimethyl disulfide was given intraperitoneally it was universally observed that a disagreeable odor suggestive of mercaptan soon became evident. On close examination it was apparent that this smell did not emanate from the site of the injection but arose from the respiratory tract. Other experimental rats were therefore placed in a closed chamber from which samples of the expired gases could be obtained. The expired air was passed through an absorption train containing lead acetate in order to absorb any hydrogen sulfide that might be present. Samples of such air were obtained from the same animal before and after intraperitoneal administration of the disulfide. It was consistently observed that the gases obtained in the latter case invariably produced a red color when passed through an alkaline solution of sodium nitroprusside, and in addition gave a red precipitate when bubbled through a solution of palladium chloride. These phenomena were not observed in the fore period (*i.e.*, prior to the administration of the disulfide). These observations suggest the presence of mercaptan (11), but characterization of the substance responsible for these reactions was not accomplished because

TABLE III

Influence of S-Methylisothiourea and Methylxanthogenate on Liver Lipid Content

The duration of the experiment was 14 days in Series I and J, 15 days in Series H, and 16 days in Series K. The amount of supplement indicated represents the quantity administered intraperitoneally during the entire period. The letters and roman numerals in the second column refer respectively to the litter and pair used in paired feeding Liver lipids are calculated on the fresh basis.

Series	Rat No.	Supplement added		Initial weight	Gain in weight	Food intake	Liver lipids
			mg	*gm*	*gm*	*gm*	*per cent*
H	I-62-p	S-Methylisothiourea	0	99	27	76	18.5
	I-63-p	"	225	92	21	73	9.6
	II-64-p	"	0	95	32	87	18.3
	II-65-p	"	225	99	34	87	10.8
	III-66-q	"	0	125	35	112	13.8
	III-67-q	"	225	113	23	112	8.1
	IV-68-q	"	0	108	39	108	15.4
	IV-69-q	"	225	107	18	107	7.9
	V-70-q	"	0	114	15	92	14.2
	V-71-q	"	225	110	19	92	8.2
I	VI-72-r	"	0	141	44	130	15.9
	VI-73-r	"	210	131	29	130	19.7
	VII-74-s	"	0	132	34	123	21.4
	VII-75-s	"	210	139	34	123	16.5
	VIII-76-s	"	0	135	24	115	24.4
	VIII-77-s	"	210	128	34	115	9.7
	IX-78-t	"	0	141	32	110	13.5
	IX-79-t	"	210	123	23	106	7.8
J	I-95-u	Methylxanthogenate	0	134	31	110	17.2
	I-96-u	"	105	115	33	111	10.2
	II-97-u	"	0	136	36	111	14.3
	II-98-u	"	105	122	18	108	9.9
	III-99-v	"	0	139	29	110	21.7
	III-100-v	"	105	153	27	110	9.6
	IV-101-v	"	0	124	28	96	12.6
	IV-102-v	"	105	119	26	94	13.7
	V-103-w	"	0	135	34	119	18.7
	V-104-w	"	105	135	39	119	19.3
K	VI-105-x	"	0	197	11	133	23.8
	VI-106-x	"	160	194	6	133	8.0
	VII-107-y	"	0	128	32	129	14.8
	VII-108-y	"	120	127	14	130	5.0
	VIII-109-z	"	0	123	19	119	18.8
	VIII-110-z	"	120	106	6	118	6.4

of the small quantities of the precipitate obtained. Nevertheless these observations suggest that a methylmercaptan might have been split off following the injections of the disulfide, and the question naturally arises whether the formation of this substance may explain the lipotropic action of this disulfide.

In view of the above findings it seemed desirable to investigate other compounds which might give rise to methylmercaptan. S-Methylisothiourea and methylxanthogenate were chosen for this purpose. According to the literature mercaptan can be liberated from the former by the action of alkali ((12) p. 841) and from the latter by simple hydrolysis ((12)-p. 843). The ester was prepared according to the procedure of Whitmore and Leiber (13) and the S-methylisothiourea by the method of Windus and Shildneck (14). As shown in Table III, both of these products are lipotropic. In

TABLE IV

Influence of Dimethyl Sulfide and Trimethylsulfonium Chloride on Liver Lipid Content

The duration of the experiment was 14 days in Series L and 15 days in Series M and N. The amount of supplement indicated represents the quantity administered during the whole period. The sulfide was given intraperitoneally; the sulfonium chloride was added to the diet. The letters and roman numerals in the second column refer respectively to the litter and pair used in paired feeding. Liver lipids are calculated on the fresh basis.

Series	Rat No	Supplement added		Initial weight	Gain in weight	Food intake	Liver lipids
			mg.	*gm.*	*gm.*	*gm.*	*per cent*
L	I-26-l	Trimethylsulfonium chloride	0	72	10	75	10.8
	I-27-l	" "	225	61	5	75	14.8
	II-28-m	" "	0	74	13	75	15.1
	II-29-m	" "	225	68	4	75	25.3
	III-30-n	" "	0	73	12	72	12.1
	III-31-n	" "	225	74	7	71	11.7
	IV-32-o	" "	0	78	12	76	15.0
	IV-33-o	" "	225	74	7	76	15.7
M	I-80-g	Dimethyl sulfide	0	91	21	79	17.4
	I-81-g	" "	563	89	17	80	13.4
	II-82-g	" "	0	84	20	82	21.2
	II-83-g	" "	563	87	9	80	8.8
	III-84-g	" "	0	100	16	96	14.5
	III-85-g	" "	563	100	17	96	13.8
	IV-86-h	" "	0	138	24	110	21.8
	IV-87-h	" "	563	149	21	107	7.8
N	V-88-i	" "	0	115	23	82	19.6
	V-89-i	" "	420	110	17	81	20.6
	VI-90-j	" "	0	118	17	69	10.3
	VI-91-j	" "	420	132	10	71	6.1
	VII-92-j	" "	0	164	52	120	13.2
	VII-93-j	" "	525	162	52	120	8.3

eight of the nine pairs, the rat receiving S-methylisothiourea had smaller amounts of liver "fat" than its paired control; the average value of 10.8 per cent for the experimental rats is significantly lower than the mean value of 17.3 per cent for the controls. Likewise in six of the eight pairs, the rat receiving methylxanthogenate had less liver "fat" than its control mate. In contrast with an average value of 16.7 per cent for the controls, the value for the animals receiving the ester was only 9.6 per cent. In view of the similarity in the behavior of dimethyl disulfide and the two products just discussed it is possible that the lipotropic action of all three of these compounds may be ascribed to the formation of methylmercaptan.

Table IV summarizes our findings with dimethyl sulfide and trimethylsulfonium chloride. The former, a commercial product (Eastman), was given intraperitoneally, but the latter proved to be so toxic, when administered intraperitoneally, that it was necessary to incorporate it in the diet. The method of Cahours (15) was used in the synthesis of the sulfonium chloride. Table IV shows that the sulfide is definitely lipotropic, since with only one exception the liver lipid content of the rats receiving this thio ether was less than that of their respective paired controls. The average values in this experiment (Series M and N) for the experimental and control animals were 11.3 per cent and 16.9 per cent, respectively. On the other hand it is clear from the data in Series L that the sulfonium compound did not exhibit lipotropic action. Unfortunately this product was so toxic that only small amounts could be given and these were of necessity added to the diet. Our interest in this compound arose from Bennett's claim (8) that methioninesulfonium chloride can be substituted for methionine for purposes of growth in the white rat.

SUMMARY

1. The intraperitoneal administration of inorganic sulfide (17 to 32 mg. of Na_2S during an experimental period of approximately 3 weeks) does not cause an accumulation of additional amounts of "fat" in the livers of young male rats fed a basal diet which produces fatty livers.

2. Dimethyl sulfide, dimethyl disulfide, S-methylisothiourea, and methylxanthogenate all exert a lipotropic effect when administered intraperitoneally to young male rats on a basal diet which produces fatty livers. Under these same circumstances neither the intraperitoneal injection of methionine sulfone nor the oral administration of trimethylsulfonium chloride caused a drop in liver lipid content.

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THE CREATINE CONTENT OF THE GASTROCNEMIUS MUSCLE OF YOUNG MALE RATS ON DIETS VARYING IN CHOLINE CONTENT

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(Received for publication, April 21, 1944)

As a result of their studies on biological methylation, du Vigneaud and coworkers (1) have suggested that substances containing physiologically labile methyl groups must be included in the diet of the rat for the synthesis of certain important methylated products such as creatine. In our various investigations on the lipotropic action of methionine and related substances (2–5), diets deficient in choline and allied products were fed, and, while we have observed many instances of acute fatty livers, no signs of creatine deficiency, such as muscular weakness or atrophy, were ever encountered. In some of the experiments very young rats were used and the experimental period included 60 days. It seemed desirable to throw more light on this question by determining the creatine content of the gastrocnemius muscle of rats in which fatty livers had been produced by diet. Young male rats were placed on a ration consisting of 5 per cent casein (Labco), 47 per cent glucose, 40 per cent lard, 5 per cent salt mixture (6), and 3 per cent Celluration (Fisher). In addition each rat received 2 drops of cod liver oil and 1 dry yeast tablet (500 mg.) daily. This ration has been used repeatedly by us for the production of fatty livers. Some of the rats received in addition choline chloride, others were given ethanolamine, while others were fed the basal ration only. In addition four rats were kept on our regular stock diet (Rockland rat diet) for 21 days. The amounts of supplements added are indicated in Table I. Ethanolamine was included because it is known to give rise to choline in the rat (7). Methyl groups are required for this transformation as well as for the synthesis of creatine. It seemed likely that methyl groups, which are ordinarily used for the production of creatine, might be diverted to the path leading to the synthesis of choline during this acute need for lipotropic substances. It is possible that this diversion might be reflected in a lowering of the muscle creatine.

The data obtained from rats (weight, 90 to 100 gm.) on these diets are summarized in Table I. Rats 1 to 7 inclusive were maintained on the respective diets indicated for 3 weeks; in the remaining cases, the period was 24 days. At the end of the experiments, the rats were decapitated and the liver and gastrocnemius muscle of one leg removed. Creatine was estimated according to Miller, Allinson, and Baker (8) without resorting to the use of enzymes to correct for the non-creatine chromogens, since 96 per cent of the total chromogenic material in this muscle of the rat is reported to be creatine (9). The intensity of the color was determined photoelectrically. The procedure for total lipids is described elsewhere (2).

TABLE I

Influence of Dietary Choline and Ethanolamine on Liver Lipid Content and Creatine Content of Gastrocnemius Muscle of Young Male Rats

The changes in body weight are those for the entire period, which consisted of 21 days in Series A and 24 days in Series B. When choline chloride was added, it replaced 0.2 per cent of glucose When ethanolamine was given, 120 mg were mixed with the diet daily The values for liver lipids and muscle creatine are calculated on the fresh basis

Series	Rat No	Supplement added	Change in weight	Food intake	Liver lipids	Muscle creatine*
			gm	*gm.*	*per cent*	*mg per 100 gm.*
A	1	Choline chloride	−5	121	5.3	443
	2	" "	+8	192	6.5	354
	3	" "	+8	181	10.4	432
	4	" "	−6	182	7.9	458
	5	None	−3	185	14.2	432
	6	"	+6	186	28.9	435
	7	"	+19	186	31.2	447
B	8	Ethanolamine	+5	180	26.2	362
	9	"	−4	156	28.2	445
	10	"	−9	170	30.6	435
	11	"	−4	188	28.4	474
	12	"	−9	190	29.5	481
	13	"	+5	187	33.6	464
	14	None	+9	182	26.8	375
	15	"	+18	159	30.6	410
	16	"	+1	171	30.5	435
	17	"	+6	188	26.6	446
	18	"	+4	189	31.9	480
	19	"	+10	185	35.8	458

* The creatine content in gastrocnemius muscle of four young male rats that had been placed on our stock Rockland rat diet for 21 days was 458, 438, 459, and 481 mg., respectively.

As shown in Table I, the supplementary choline afforded almost complete protection against the infiltration of unusual amounts of "fat" into the livers. On the other hand the control animals (*i.e.*, those receiving the unsupplemented diet) and those given ethanolamine as a supplement all exhibited fatty livers. The livers of the four rats receiving the Rockland rat diet were not analyzed for total lipids, but upon inspection these organs appeared perfectly normal. The average muscle creatine value of these four animals (457 mg. per cent) compares well with the mean value of 422 mg. per cent for the rats receiving the supplement of choline. The corresponding values of 435 for the control rats and 444 for those receiving ethanolamine as a supplement are in close agreement with other values cited above. It is obvious thus that, even though a choline deficiency was evident in Rats 5 to 19 inclusive, the average creatine content of the gastrocnemius muscle of these animals was not affected by this deficiency. Dietary ethanolamine was evidently without influence on liver lipid or gastrocnemius muscle content. The remaining data in Table I indicate that the creatine content of this particular muscle was not related to either the food intake or changes in body weight. After this work had been completed, it was reported that a deficiency in dietary choline does not appreciably diminish creatine formation in the chick (10).

SUMMARY

The creatine content of the gastrocnemius muscle of young white rats (male, 90 to 100 gm.) is not lowered when a diet deficient in choline is fed for a period of 3 weeks. The addition of ethanolamine to this diet is without influence on the creatine content of this muscle.

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INHIBITING EFFECT OF INORGANIC IODIDE ON THE FORMATION IN VITRO OF THYROXINE AND DIIODOTYROSINE BY SURVIVING THYROID TISSUE*

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(Received for publication, April 17, 1944)

When 300 mg. of surviving thyroid slices are incubated for 2 to 3 hours in a Ringer's medium containing radioactive iodide, it is found that as much as 60 per cent of the labeled iodine is organically bound, about 50 per cent as diiodotyrosine and about 10 per cent as thyroxine (1). This procedure has been used to study the mechanism of the formation of thyroxine and diiodotyrosine by the thyroid gland. Thus it was shown that the synthesis of these compounds by thyroid tissue is linked with aerobic oxidations in which the cytochrome-cytochrome oxidase system participates (2).

The present investigation deals with the formation *in vitro* of thyroxine and diiodotyrosine by thyroid tissue from added inorganic iodide in cases in which the inorganic iodide (I^{127}) added to the reaction flasks containing 300 mg. of thyroid tissue was varied from 0 to 50 γ. It is shown here that the conversion of the added iodide to thyroxine and diiodotyrosine is inhibited when the amount of inorganic iodide in the medium surrounding the thyroid slices exceeds 20 γ.

EXPERIMENTAL

The preparation of slices of thyroid gland of the sheep has been described elsewhere (1). As a rule, slices were prepared from ten glands and placed in a large Petri dish containing a bicarbonate-Ringer's solution. Slices were selected at random, blotted on filter paper, quickly weighed, and transferred to a 25 cc. Erlenmeyer flask containing 3 cc. of a bicarbonate-Ringer's solution. The bicarbonate-Ringer's medium used was essentially the same as that described by Krebs and Henseleit (3). The iodide I^{131} was prepared in a small volume of isotonic sodium chloride, and this was added to the stock solution of isotonic sodium chloride used in the preparation of the medium. The 3 cc. of medium contained in each reaction flask were saturated with a gas mixture consisting of 5 per cent carbon dioxide and 95 per cent oxygen before the addition of the thyroid slices; the atmosphere above the solution and slices was displaced with this same gas

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* Aided by grants from the Commonwealth Fund and the Committee for Research in Endocrinology of the National Research Council.

mixture immediately before the flask was placed in the constant temperature water bath and again after the 1st hour of incubation. The flasks were gently agitated during their 2 hour stay in a constant temperature bath maintained at 38°.

The I^{127} content of the medium was varied by adding to each 3 cc. of bicarbonate-Ringer's solution contained in the reaction flask the required amount of potassium iodide dissolved in 0.1 cc. of distilled water. It is estimated that the I^{127} in the bicarbonate-Ringer's solution due to impurities in the reagent quality salts used in its preparation amounted to 0.1 γ per cc.

The entire contents of the flask were analyzed for thyroxine I^{131}, diiodotyrosine I^{131}, and inorganic iodide I^{131} according to a procedure described elsewhere (1). Each of these fractions in Experiments 1 and 2 was independently measured; in Experiments 3, 4, and 5 inorganic iodide was obtained by difference.

Results

The results shown in each table represent a single experiment in which the thyroid slices for its six to twelve flasks were selected at random from a single batch of slices prepared from several sheep glands at one time. The amount of thyroid tissue added to each flask was kept as constant as possible; the amounts added varied from 300 to 321 mg. The iodide I^{127} present in the flasks was varied from 0.3 to 50.3 γ; 0.3 γ was the amount present in each 3 cc. of bicarbonate-Ringer's solution as an unavoidable impurity.

The recoveries of the added I^{131} in the form of thyroxine, diiodotyrosine, and inorganic iodide are recorded in Columns 4, 5, and 6 of each table. The micrograms of Ringer's I^{127} converted to thyroxine in each flask were obtained by multiplying the value shown in Column 3 by the respective value shown in Column 4; the micrograms of Ringer's I^{127} converted to diiodotyrosine were obtained by multiplying the value in Column 3 by the value in Column 5. These calculated values are recorded in Columns 7 and 8.

Experiment 1 (Table I) shows that, as the concentration of I^{127} in the medium surrounding the thyroid slices was increased from 0.3 to 10 γ, the amounts of the Ringer's I^{127} converted to thyroxine and diiodotyrosine also increased. When, however, the I^{127} content of the Ringer's medium was raised to 20 and 50 γ, less of it was organically bound in these two compounds than when the medium of the reaction flask contained only 10 γ. Thus when 300 mg. of thyroid slices were deposited in Ringer's media containing 10.3, 20.3, and 50.3 γ of I^{127}, the following amounts of the Ringer's I^{127} were converted to thyroxine: 0.82, 0.26, and 0.12 γ respectively;

TABLE I

Experiment 1

Flask No.	Thyroid tissue in flask	Inorganic iodide I^{127} added to flask	Per cent of Ringer's I^{131} recovered as			Ringer's I^{127} converted to	
			Thyroxine	Diiodotyrosine	Inorganic	Thyroxine	Diiodotyrosine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mg.	γ				γ	γ
1	307	0.3*	7.3	73.2	18.1	0.02	0.22
2	306	0.3*	7.3	66.8	22.2	0.02	0.20
3	321	1.3	8.2	61.7	23.4	0.11	0.80
4	314	1.3	8.5	61.8	22.3	0.11	0.80
5	305	5.3	5.9	43.7	44.9	0.31	2.3
6	300	5.3	5.0	43.4	46.7	0.27	2.3
7	300	10.3	8.2	49.2		0.84	5.1
8	309	10.3	7.7	42.8	39.6	0.79	4.4
9	314	20.3	1.4	11.7	84.0	0.29	2.4
10	309	20.3	1.2	10.3	83.5	0.24	2.1
11	312	50.3	0.30	1.6	98.4	0.15	0.80
12	311	50.3	0.20	3.0	92.2	0.10	1.5

* These amounts were not added to the Ringer's medium; their presence was due to impurities in the reagent grade chemicals used in the preparation of the media.

TABLE II

Experiment 2

Flask No	Thyroid tissue in flask	Inorganic iodide I^{127} added to flask	Per cent of Ringer's I^{131} recovered as			Ringer's I^{127} converted to	
			Thyroxine	Diiodotyrosine	Inorganic	Thyroxine	Diiodotyrosine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mg	γ				γ	γ
1	302	0.3*	9.4	45.0	42.2	0.03	0.14
2	304	0.3*	10.9	47.6	41.2	0.03	0.14
3	300	5.3	9.9	47.7	40.9	0.53	2.5
4	303	5.3	8.6	36.0	56.0	0.46	1.9
5	302	10.3	5.3	22.8	72.0	0.55	2.3
6	302	10.3	6.4	24.8	68.2	0.66	2.6
7	304	15.3	4.8	17.5	74.5	0.73	2.7
8	300	15.3	5.5	19.9	76.6	0.84	3.0
9	304	20.3	4.5	16.6	78.5	0.91	3.4
10	303	20.3	6.1	18.4	75.5	1.24	3.7

* See foot-note to Table I.

the amounts converted to diiodotyrosine were 4.8, 2.2, and 1.1 γ respectively. These values are the averages of those recorded in Table I.

No inhibitory effect was observed in Experiment 2 (Table II), but in this experiment the amounts of I^{127} added to the reaction flask did not exceed 20 γ.

TABLE III

Experiment 3

Flask No.	Thyroid tissue in flask	Inorganic iodide I^{127} added to flask	Per cent of Ringer's I^{131} recovered as			Ringer's I^{127} converted to	
			Thyroxine	Diiodotyrosine	Inorganic	Thyroxine	Diiodotyrosine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mg.	γ				γ	γ
1	305	0.3*	10.4	65.3	24.3	0.03	0.20
2	300	0.3*	11.8	70.2	18.0	0.04	0.21
3	301	0.3*	10.9	69.6	19.5	0.03	0.21
4	302	10.3	11.1	59.0	29.9	1.1	6.1
5	300	10.3	7.7	45.2	47.1	0.79	4.7
6	303	10.3	9.2	50.0	40.8	0.95	5.1
7	306	20.3	4.9	29.6	65.5	1.0	6.0
8	301	20.3	5.9	33.7	60.4	1.2	6.8
9	302	20.3	6.3	31.3	62.4	1.3	6.4
10	305	50.3	1.7	9.7	88.6	0.85	4.9
11	302	50.3	0.80	6.5	92.7	0.40	3.3
12	305	50.3	0.60	5.9	93.5	0.30	3.0

* See foot-note to Table I.

TABLE IV

Experiment 4

Flask No.	Thyroid tissue in flask	Inorganic iodide I^{127} added to flask	Per cent of Ringer's I^{131} recovered as			Ringer's I^{127} converted to	
			Thyroxine	Diiodotyrosine	Inorganic	Thyroxine	Diiodotyrosine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mg	γ				γ	γ
1	310	0.3*	8.6	72.9	18.5	0.03	0.22
2	301	0.3*	8.4	77.0	14.6	0.03	0.23
3	301	5.3	7.4	56.6	36.0	0.39	3.0
4	305	5.3	5.2	49.3	45.5	0.28	2.6
5	313	10.3	3.9	31.1	65.0	0.40	3.2
6	300	10.3	3.9	25.9	70.2	0.40	2.7
7	300	20.3	2.3	15.7	82.0	0.47	3.2
8	300	20.3	2.3	16.6	81.1	0.47	3.4
9	305	50.3	0.50	2.5	97.0	0.25	1.3
10	304	50.3	0.50	3.7	95.8	0.25	1.9

* See foot-note to Table I.

Experiments 3 and 4 (Tables III and IV) bring out quite well the inhibitory action of inorganic iodide. In both experiments the largest amounts of Ringer's I^{127} converted to thyroxine and diiodotyrosine occurred in those cases in which 20 γ had been added to the medium surrounding the thyroid slices. From 1.0 to 1.3 γ of Ringer's I^{127} was incorporated into thyroxine when 20 γ of I^{127} had been added (Experiment 3). As little as 0.3 γ was converted to thyroxine when 50 γ had been added. In Experiment 4, the amounts of Ringer's I^{127} converted to thyroxine were cut in half when the concentration of I^{127} in the medium was raised from 20 to 50 γ. A decreased conversion of inorganic iodide to diiodotyrosine was also found under the conditions mentioned above.

When the concentration of Ringer's I^{127} was changed from 20.3 to 50.3 γ in Experiment 5 (Table V), thyroxine formation at the expense of Ringer's I^{127} was reduced to about the same extent as that observed in Experiment 4.

TABLE V

Experiment 5

Flask No (1)	Thyroid tissue in flask (2)	Inorganic iodide I^{127} added to flask (3)	Per cent of Ringer's I^{131} recovered as			Ringer's I^{127} converted to	
			Thyroxine (4)	Diiodo-tyrosine (5)	Inorganic (6)	Thyroxine (7)	Diiodo-tyrosine (8)
	mg	γ				γ	γ
1	300	0.3*	10.6	45.6	43.8	0.03	0.14
2	300	0.3*	8.7	42.9	48.4	0.03	0.13
3	305	20.3	2.2	6.1	91.7	0.45	1.2
4	305	20.3	2.1	6.9	91.0	0.43	1.4
5	305	50.3	0.50	2.4	97.1	0.25	1.2
6	300	50.3	0.40	2.4	97.2	0.20	1.2

* See foot-note to Table I.

The amounts of Ringer's I^{127} converted to diiodotyrosine were about the same in the samples containing 20 and 50 γ.

DISCUSSION

The concentration of added inorganic iodide at which inhibitory effects upon the conversion of inorganic iodide (in the medium surrounding the thyroid slice) to thyroxine and diiodotyrosine were observed was not the same in the five experiments. Definite inhibition was found in Experiments 1, 3, 4, and 5. In Experiment 1, inhibition was observed between 10 and 20 γ. In Experiment 2, in which the amounts of I^{127} added did not exceed 20 γ, no inhibition was noted. In Experiments 3 and 4 inhibitory effects were not observed before the 50 γ level. Such differences are not surprising, for it is not to be expected that the thyroid glands, although uniform with respect to the various samples of a single experiment, were uniform throughout all five experiments with respect to their iodine content or functional activity. Not only were the sheep obtained from different parts of the country, but the experiments were conducted at different times of the year.

The data recorded in Tables I to V furnish no information as to the amounts of thyroxine and diiodotyrosine formed from their organic precursors present inside the slice at the time the gland was excised from the animal. If, however, none is formed from these precursors, the values shown in Columns 7 and 8 of the tables represent the total amounts of thyroxine and diiodotyrosine synthesized during the course of the experiment. If, as seems more likely, iodine present in the gland before it was put into the reaction flask is also incorporated into newly formed thyroxine and diiodotyrosine, then the amounts shown in Columns 7 and 8 are less than the total amounts formed.

Various explanations of the inhibitory effects of iodide observed here are considered below.

Penetration of Ringer's Iodide into Thyroid Slice (Permeability)—The rate of transport of the outside iodide to the actual sites of thyroxine and diiodotyrosine formation inside the thyroid slice is obviously a factor involved in the amounts of thyroxine and diiodotyrosine formed in the 2 hour period of incubation. This raised the question whether the inhibitory effect observed here could result from a diminished amount of iodide reaching the site of reaction. To assume that the inhibitory effect is due to a diminished penetration of iodide would involve the inference, untenable at first thought, that the greater the concentration of iodide in the medium surrounding the thyroid slice (between 10 and 50 γ) the less iodide reaches the site of reaction. Schachner has measured the amounts of I^{127} that entered surviving thyroid slices when the Ringer's media contained 10, 20, and 50 γ of inorganic iodide.[1] The procedure for this type of measurement has been described elsewhere (4). The largest amounts of Ringer's I^{127} entered the slices that were incubated in the media containing 50 γ of I^{127}.

Iodination of Enzymes Concerned with Conversion of Inorganic Iodide to Thyroxine and Diiodotyrosine—Herriott has shown that iodine inactivates pepsin by reacting with the tyrosine of this enzyme (5). After complete iodination he was able to recover over 80 per cent of the iodine as diiodotyrosine. These findings suggest that the inhibitory effects observed here on the formation *in vitro* of thyroxine and diiodotyrosine are brought about by iodination of an enzyme or enzymes concerned with the conversion of inorganic iodide to thyroxine and diiodotyrosine. If this should be true, inorganic iodide would serve not only as a precursor of thyroxine and diiodotyrosine but also as an agent for regulating the concentration of the enzyme in thyroid tissue concerned with their formation.

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[1] Schachner, H., unpublished observations.

Inhibition of Formation of an Intermediate in Synthesis of Thyroxine and Diiodotyrosine—The presence of increased concentrations of organic iodide may serve to depress the formation of a substance intermediate in the conversion of inorganic iodide to thyroxine and diiodotyrosine. In the scheme of Johnson and Tewkesbury, hypoiodous acid (HIO) is proposed as an intermediate in the formation of thyroxine (6). If this is assumed to be true, the inhibition of the formation of an intermediate by iodide may be visualized as follows:

$$I^- + \text{oxidizing agent} \rightarrow I_2 \qquad (1)$$

$$I_2 + H_2O \rightleftarrows HIO + H^+ + I^- \qquad (2)$$

In any system in which the amount of I_2 formed in Equation 1 is not a function of the amount of I^- present (*e.g.* the amount of I_2 formed would be limited if the specific oxidizing agent were used up by I^-) the presence of excess I^- would decrease the formation of HIO in the scheme shown in Equation 2.

It is of interest to note here that Li has shown that the rate of iodination of tyrosine at pH 7.4 is mainly dependent upon the concentration of hypoiodous acid (7). From kinetic studies, he was also able to demonstrate that inorganic iodide inhibits the formation of diiodotyrosine.

SUMMARY

1. 300 mg. of thyroid slices were incubated in a bicarbonate-Ringer's medium to which 1, 5, 10, 15, 20, and 50 γ of I^{127} as inorganic iodide were added. The inorganic iodide of the medium was labeled with radioactive iodine (I^{131}). The recovery of radiothyroxine and radiodiiodotyrosine therefore served to measure the amounts of the medium's I^{127} converted to these two compounds.

2. Inorganic iodide inhibited the formation of thyroxine and diiodotyrosine at the expense of inorganic iodide of the medium. An inhibitory effect on the conversion of Ringer's I^{127} to thyroxine and diiodotyrosine was observed in one experiment between the levels of 10 and 20 γ and in three experiments between 20 and 50 γ.

3. Possible mechanisms by which inorganic iodide inhibits the formation of thyroxine and diiodotyrosine are discussed.

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MERCAPTURIC ACIDS

V. THE METABOLIC FORMATION OF p-FLUOROPHENYLMERCAPTURIC ACID FROM FLUOROBENZENE*

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(Received for publication, April 19, 1944)

The question of whether or not fluorobenzene shares with chlorobenzene (2), bromobenzene (3), and iodobenzene (4) the property of giving rise to mercapturic acid formation in the animal body has hitherto remained unanswered. From the work of Coombs (5) it is known that the administration of fluorobenzene has an effect on the sulfur metabolism of the dog. Coombs observed that when the compound was given to dogs either subcutaneously or by mouth it brought about a rise in the neutral sulfur content of the urine. He pointed out, however, that this increase was small compared with that produced by the other monohalogen-substituted benzenes under similar conditions. Coombs noted that shortly after the administration of fluorobenzene this compound could easily be detected by its odor in the breath of the animal, and he suggested that the disparity in the effects produced by fluorobenzene and chlorobenzene on sulfur excretion might be related in part to the greater volatility of fluorobenzene. An increase in neutral sulfur excretion following the administration of a foreign organic compound is of uncertain value as evidence of mercapturic acid excretion, and as no work directed towards the isolation of a mercapturic acid from the urine of animals dosed with fluorobenzene appears to have been reported, the investigation described herein was undertaken.

In Paper IV of this series (6) the synthesis of *p*-fluorophenylmercapturic acid was described and an account was given of the isolation of this compound from the urine of rats which had ingested *p*-fluorophenyl-*l*-cysteine. In the present work it has been possible to show that fluorobenzene is converted to a mercapturic acid in the rat. Fluorobenzene was administered to rats by stomach tube or by subcutaneous injection and from the urine of the dosed animals a compound was isolated which was identified as *p*-fluorophenylmercapturic acid on the basis of its analysis and a comparison of its properties with those of the synthetic compound.

EXPERIMENTAL

The sample of fluorobenzene (Eastman Kodak Company) which was used in the present work boiled at 84–85°. *p*-Fluorophenylmercapturic acid was synthesized by the method described in Paper IV of this series (6). It melted[1] at 158–159° and for a 1 per cent solution in ethanol its specific rotation was $[\alpha]_D^{24} = -20°$.

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* A preliminary report of the work described herein was presented at a meeting of the Toronto Biochemical and Biophysical Society held on February 17, 1944 (1).

Isolation of p-Fluorophenylmercapturic Acid from Urine—The method used to isolate *p*-fluorophenylmercapturic acid from the urine of rats dosed with fluorobenzene was similar in principle to that used to isolate this compound from the urine of rats which had ingested *p*-fluorophenyl-*l*-cysteine (6). The two main steps in the procedure were the repeated extraction of the acidified urine with chloroform and the separation of the mercapturic acid from the combined chloroform extracts.

The urine was made acid to Congo red by the addition of concentrated hydrochloric acid and was allowed to stand overnight. It was then extracted six times with portions of chloroform, each equal in volume to that of the acidified urine. Each portion of chloroform was shaken vigorously with the urine for 10 minutes and the emulsion which formed was broken by centrifuging. The chloroform extracts were filtered, combined, and allowed to stand over anhydrous sodium sulfate. The dried chloroform solution was filtered and reduced to a small volume by distillation on a water bath. The remaining chloroform was then removed by evaporation under reduced pressure and a dark oily residue which contained some crystalline material was obtained. The whole residue was dissolved in a few ml. of chloroform which had been thoroughly washed with water, dried, and distilled immediately before use. The chloroform solution was concentrated to a small volume and cooled in a freezing mixture. The crystalline precipitate which separated was filtered and washed with small portions of cold chloroform. It was then dissolved in ethanol and the solution was decolorized with charcoal, filtered, and evaporated to dryness. The residue was crystallized from aqueous ethanol and colorless crystals of pure *p*-fluorophenylmercapturic acid were obtained.

The isolation procedure described above differed from that used previously (6) in that the urine was extracted with six, instead of three, portions of chloroform. The need for more than three extractions with chloroform was first suggested by the results of two recovery experiments. In each of these experiments 0.100 gm. of synthetic *p*-fluorophenylmercapturic acid was dissolved in 100 ml. of normal rat urine. The urine was then treated as described above except that in the first experiment it was extracted three times with chloroform and in the second experiment six extractions with chloroform were made. The amount of pure mercapturic acid recovered was 0.067 gm. in the first experiment and in the second it was 0.082 gm. In two experiments in which the mercapturic acid was isolated from the urine of rats dosed with fluorobenzene nine chloroform extractions were performed. In both cases the first three extracts were found to contain most of the mercapturic acid, the second three extracts contained a small quantity, and the third three extracts did not contain a significant amount of the compound.

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[1] All melting points reported herein are uncorrected.

In the isolation of *p*-fluorophenylmercapturic acid from the urine of rats which had ingested *p*-fluorophenyl-*l*-cysteine (6) the residue obtained on evaporation of the chloroform extracts consisted of little more than the mercapturic acid and readily yielded the pure compound on crystallization from aqueous ethanol. On the other hand, the evaporation of the chloroform extracts of the urine of rats dosed with fluorobenzene yielded a residue which contained a high proportion of oily material. It was found, however, that the mercapturic acid could be separated from the oil by making use of the high solubility of the oil and the low solubility of the mercapturic acid in cold chloroform.

In order to test the effectiveness of the isolation process employed in the present work a recovery experiment was performed with urine collected from a group of rats which had been dosed with fluorobenzene by subcutaneous injection. From a 100 ml. portion of this urine 0.114 gm. of pure mercapturic acid was isolated. To another 100 ml. portion of this urine was added a neutralized solution (2 ml.) of 0.050 gm. of synthetic *p*-fluorophenylmercapturic acid. This urine yielded 0.161 gm. of mercapturic acid, which represents a recovery of 94 per cent of the added compound.

Excretion of p-Fluorophenylmercapturic Acid Following Administration of Fluorobenzene to Rats by Stomach Tube—Male white rats were used in the experiments in which fluorobenzene was administered by stomach tube. The animals were housed in metabolism cages which permitted the collection of urine separate from the feces. They were fed on a diet which consisted of Master Fox Breeding Ration (Toronto Elevators Limited) supplemented with fresh milk daily and whole wheat bread twice a week. In order to avoid contamination of the urine with fallen food the rats were fed in a separate cage twice daily for periods of 1 hour each, and the urine excreted during these periods was not collected. The animals had access to drinking water at all times. The urine excreted by the rats while they were in the metabolism cages was collected daily from the time of administration of the first dose of fluorobenzene until 2 days after the last dose of the compound had been given. The collected urine was stored in the refrigerator.

Six rats were each given 0.25 ml. of fluorobenzene by stomach tube daily for 4 days and during this period their average body weight fell from 273 gm. to 232 gm. In the same period the average body weight of a group of six undosed rats which were fed and housed under the same conditions fell from 266 gm. to 256 gm. The dosed animals received a total of 6.1 gm. of fluorobenzene and when the isolation procedure described above was applied to the urine 0.202 gm. of a colorless crystalline product was obtained. This material melted at 158–159° and when it was mixed with synthetic *p*-fluorophenylmercapturic acid, m.p. 158–159°, the melting point was unchanged. When analyzed the compound yielded the following results.

$C_{11}H_{12}O_3FNS$.	Calculated.	C 51.33, H 4.70, N 5.45, S 12.47
	Found.	" 51.29, " 4.47, " 5.56, " 12.80

The specific rotation of a 1 per cent solution of the compound in ethanol was $[\alpha]_D^{26} = -19°$.

The determination of its equivalent weight by titration with 0.01 N sodium hydroxide solution yielded 260. The calculated equivalent weight of *p*-fluorophenylmercapturic acid is 257.

On the basis of these various findings it appeared that the compound isolated from the urine in the above experiment was *p*-fluorophenylmercapturic acid. This was supported by the results of experiments (described later) in which the compound was submitted to decomposition by acid and by alkali.

A second group of six rats was dosed with fluorobenzene by stomach tube under the conditions of the first experiment. Each rat received 0.25 ml. of fluorobenzene daily for 4 days. The animals received a total of 6.1 gm. of fluorobenzene and from their urine 0.312 gm. of crystalline product was obtained. This material melted at 158–159°, and had a specific rotation of $[\alpha]_D^{25} = -19°$ for a 1 per cent solution in ethanol. During the experiment the average weight of the animals in the group fell from 268 gm. to 238 gm.

Excretion of p-Fluorophenylmercapturic Acid Following Administration of Fluorobenzene to Rats by Subcutaneous Injection—The conditions of housing and feeding the rats and the collection of urine were the same in the experiments in which fluorobenzene was injected subcutaneously as in those in which the compound was given by stomach tube. Male white rats were used in the experiments.

In an experiment with six rats 0.25 ml. of fluorobenzene was injected daily for 4 days under the skin of the back of each animal. Although this amount of fluorobenzene did not kill any of the rats, they were in poor condition at the end of the dosing period and their average body weight had fallen from 288 gm. to 254 gm. These rats received a total of 6.1 gm. of fluorobenzene and from their urine 0.189 gm. of colorless crystals was isolated by means of the procedure already described. This product melted at 158–159°, and when it was mixed with synthetic *p*-fluorophenylmercapturic acid the melting point was not depressed. On analysis the isolated compound yielded the following results.

$C_{11}H_{12}O_3FNS$. Calculated. C 51.33, H 4.70, N 5.45, S 12.47
Found. " 51.44, " 4.83, " 5.46, " 12.75

The specific rotation of the compound was $[\alpha]_D^{26} = -19°$ for a 1 per cent solution in ethanol.

The conclusion that the compound obtained from the urine in the above experiment was *p*-fluorophenylmercapturic acid was supported by the results of experiments in which it was decomposed by acid and by alkali.

In a similar experiment to that just described another six rats were each given 0.25 ml. of fluorobenzene by subcutaneous injection daily for 4 days and during this period their average weight fell from 281 gm. to 254 gm. From their urine was isolated 0.349 gm. of crystalline product which melted at 158–159° and which had a specific rotation of $[\alpha]_D^{25} = -19°$ for a 1 per cent solution in ethanol.

Decomposition, by Alkali and by Acid, of Compound Isolated from Urine of Rats Dosed with Fluorobenzene—The experiments described below were performed in order to test the conclusion that the compound isolated from the urine of rats dosed with fluorobenzene was *p*-fluorophenylmercapturic acid.

A study was first made of the decomposition of synthetic *p*-fluorophenylmercapturic acid by alkali. 0.050 gm. of the synthetic compound was heated with excess of 2 N sodium hydroxide solution in a boiling water bath. Ammonia was evolved during the heating process, and when the solution was cooled and acidified with hydrochloric acid a milky precipitate formed which settled as an oil on standing. The oil was probably *p*-fluorophenyl mercaptan. It had a strong mercaptan odor, it was soluble in ether, and when dissolved in aqueous ethanol it decolorized iodine solution with the formation of an oily precipitate. In this connection it should be noted that Seyhan (7) reported that he was unable to obtain a crystalline disulfide by the oxidation of *p*-fluorophenyl mercaptan. When the compound isolated from the urine of rats dosed with fluorobenzene was subjected to experiments of the type described above, it behaved in the same manner as the synthetic mercapturic acid and no difference could be detected in the odors of the oily products which were formed on acidification of the solutions obtained after heating the isolated and synthetic compounds with an excess of 2 N sodium hydroxide solution.

Suitable conditions for the conversion of *p*-fluorophenylmercapturic acid to *p*-fluorophenyl-*l*-cysteine by acid hydrolysis were determined by means of experiments with the synthetic mercapturic acid. These conditions were then employed in the decomposition of the compound isolated from the urine of rats dosed with fluorobenzene by stomach tube. 0.162 gm. of this compound was boiled under a reflux for 1 hour with 5 ml. of 25 per cent (by volume) sulfuric acid. The solution was cooled in ice, filtered, and the filtrate was made neutral to Congo red by the addition of ammonia. The crystalline precipitate which formed was filtered, washed with water, and after being dried over phosphorus pentoxide *in vacuo* it weighed 0.097 gm. On analysis it yielded the following results.

$C_9H_{10}O_2FNS$. Calculated, C 50.20, H 4.68; found, C 50.28, H 4.70

It decomposed at 180–183° and had a specific rotation of $[\alpha]_D^{22} = +13°$ for a 1 per cent solution in 0.1 N sodium hydroxide. Synthetic *p*-fluorophenyl-*l*-cysteine decomposes at 180–183° and has a specific rotation of $[\alpha]_D^{22} = +13°$ for a 1 per cent solution in 0.1 N sodium hydroxide (6). In an experiment similar to that described above, 0.146 gm. of the compound isolated from the urine of rats dosed with fluorobenzene by subcutaneous injection was decomposed by acid. The crystalline product which was obtained weighed 0.096 gm. It showed the same decomposition point and specific rotation as synthetic *p*-fluorophenyl-*l*-cysteine and when analyzed it yielded the following results.

$C_9H_{10}O_2FNS$. Calculated, C 50.20, H 4.68; found, C 50.23, H 4.66

The results of the decomposition experiments all supported the conclusion, reached on other grounds, that the compound isolated from the urine of rats dosed with fluorobenzene was *p*-fluorophenylmercapturic acid.

DISCUSSION

A description has been given of the isolation of a crystalline compound from the urine of rats dosed with fluorobenzene by stomach tube or by subcutaneous injection. This compound has been identified as *p*-fluorophenylmercapturic acid on the basis of its analysis, melting point, melting point when mixed with synthetic *p*-fluorophenylmercapturic acid, specific rotation, behavior on decomposition with alkali, and by its conversion to *p*-fluorophenyl-*l*-cysteine by acid hydrolysis. In two experiments in which rats were given fluorobenzene by stomach tube the amounts of the mercapturic acid which were isolated corresponded to 1.2 and 1.9 per cent of the fluorobenzene administered. When fluorobenzene was injected subcutaneously into rats, 1.2 per cent of the compound was obtained from the urine in the form of the mercapturic acid in one experiment and 2.1 per cent in another. In every experiment the amount of mercapturic acid isolated was small in relation to the amount of fluorobenzene administered. It should be noted, however, that not all of the mercapturic acid excreted by the rats was isolated in these experiments. The urine excreted while the rats were in the feeding cage was not collected and a small proportion of the urine was lost in this way. Some loss of mercapturic acid also occurred in the isolation process, although the results of recovery experiments suggest that such loss was not great.

The investigations of numerous workers have established that chlorobenzene, bromobenzene, and iodobenzene are metabolized by various mammalian species in such a way as to give rise to the corresponding *p*-halogen-substituted phenylmercapturic acids. As a result of the present finding that *p*-fluorophenylmercapturic acid is synthesized from fluorobenzene in the rat, the generalization can now be made that all the monohalogen-substituted benzenes have been shown to be converted to mercapturic acids *in vivo*.

SUMMARY

p-Fluorophenylmercapturic acid has been isolated from the urine of rats following the administration of fluorobenzene by stomach tube or by subcutaneous injection. The isolated compound has been identified by analysis and by comparison of its properties with those of synthetic *p*-fluorophenylmercapturic acid.

One of us (S. H. Z.) is indebted to the Banting Research Foundation for a personal grant.

The microanalyses reported herein were performed by Mr. Michael Edson.

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INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

XVI. A SOURCE OF ERROR IN THE MANOMETRIC NINHYDRIN METHOD FOR THE ANALYSIS OF AMINO ACIDS AND ITS SUPPRESSION BY THE USE OF HYDRAZINE*

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(Received for publication, April 12, 1944)

A method for the analysis of α-amino acids based on the manometric measurement of carbon dioxide liberated by the action of ninhydrin was reported by Van Slyke, Dillon, MacFadyen, and Hamilton (2) in 1941. It was found that this method is specific for α-amino acids and gives quantitative results at some pH between 1 and 5. Improvements in the procedures were described in subsequent papers by MacFadyen (3), Hamilton and Van Slyke (4), and Van Slyke, MacFadyen, and Hamilton (5).

It has been found by the present authors that net pressures higher than the theoretical P_{CO_2} values result when several of the amino acids are analyzed by the manometric procedure described. The formation of certain aldehydes which distil into the manometric chamber and affect unequally the two pressure readings is believed to be the source of this error. A simple modification of the method devised to overcome this difficulty and a series of test data are reported in the experimental part.

EXPERIMENTAL

Apparatus—The portable Van Slyke-Neill apparatus (6) was used to measure carbon dioxide. The calibrated glass spoons and alkali storage vessels were patterned after those of Van Slyke and Folch (7) and the adapters, clamps, glass rods, and mercury bottle after those of MacFadyen (3).[1]

Reagents—Ninhydrin purchased from the University of Illinois was pulverized and stored in a dark bottle.[2] Solid citrate buffers of pH 2.5 and 4.7, 2.0 N lactic acid solution, 5.0 N sodium hydroxide solution, and nearly carbon dioxide-free 0.5 N sodium hydroxide solution were prepared according to the directions given by Van Slyke *et al.* (2), and solutions of 0.5 N sodium hydroxide and of 2.0 N lactic acid in 25 per cent sodium chloride according to the directions of MacFadyen (3). A solution of 0.5 N sodium hydroxide and 0.15 M hydrazine was prepared by dissolving 5 gm. of hydrazine sulfate in 200 to 230 ml. of carbon dioxide-free distilled water in a 250 ml. volumetric flask, cooling the mixture in ice, adding 12.2 ml. of 16.7 N sodium hydroxide solution, and adding distilled water to the mark. The solution was thoroughly mixed and transferred to a storage vessel designed to protect the alkali solution from contamination by carbon dioxide.[3] A second solution of 0.5 N sodium hydroxide and 0.15 M hydrazine was prepared in the same manner except that the reagents were made up in 25 per cent sodium chloride solution rather than distilled water.

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* For Paper XV in this series see Dunn *et al.* (1). This work was aided by grants from the Gelatin Products Company, Merck and Company, Inc., and the University of California.

[1] In some of the experiments on methionine the all-glass vessels advocated by Hamilton and Van Slyke (4) were employed.

[2] The later experiments utilized ninhydrin prepared by Mr. R. C. Bovie as a student exercise, using the procedure of Teeters and Shriner (8).

Amino Acids—An approximately 100 to 200 mg. sample of the amino acid[4] was weighed to 0.1 mg. and transferred to a 25 or 50 ml. calibrated volumetric flask. The sample was dissolved and the solution diluted to volume with sufficient distilled water or dilute hydrochloric acid. All solutions not analyzed at once were preserved in the refrigerator.

Experimental Procedures—A 1 ml. aliquot of the amino acid solution was transferred to the reaction flask with a calibrated pipette. Solid buffer (50 mg.) and, if the pH of the solution was not at the desired level, the required quantity of trisodium citrate were added. The adapter was put in place, the solution was evacuated for 2 minutes, the adapter was removed, and about 50 mg. of ninhydrin were added. The adapter was replaced and the solution was evacuated for 10 seconds. The flask was sealed (3), placed in a rapidly boiling water bath for a measured period, and transferred to a 37–39° bath. After approximately 2 minutes the flask was removed from the bath and connected immediately with the manometric apparatus (3) which previously had been charged with one of the four types of 0.5 N sodium hydroxide solution.

The carbon dioxide resulting from the reaction with ninhydrin was measured by each of the following three procedures.

Original Technique—The procedure described by Van Slyke *et al.* (2) and MacFadyen (3) for the absorption of the carbon dioxide in a 0.5 N solution of sodium hydroxide in water or in 25 per cent sodium chloride solution was followed. The complete absorption of the carbon dioxide was insured by lowering the mercury eleven times in analyses with reagents containing distilled water and six times with reagents containing 25 per cent sodium chloride solution as solvents. Following these manipulations the mercury level was raised to the middle of the chamber, the upper cock was closed, and the reaction flask was removed.[5] The remaining manipulations were carried out as described by Van Slyke *et al.* (2), except that, after the addition of the lactic acid, the apparatus was shaken for 5 minutes with the mercury level at the 50 ml. mark to equilibrate the carbon dioxide in the two phases.

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[3] This solution was essentially the same as that used by Van Slyke and Folch (7) for combustion analysis of carbon.

[4] Amino Acid Manufactures' C. P. or A. P. grade or Merck and Company's.

Double Equilibration Technique—The original technique was followed except that the residual gases and the alkaline lactate solution were equilibrated with the mercury level at the 50 ml. mark before the pressure (p_2) was read at 2 ml. volume. In practice the p_2 value measured by the "double equilibration technique" was usually determined immediately after the p_2 value by the "original technique" had been recorded.

Hydrazine Technique—This technique differed from the "original technique" only in that a 0.5 N sodium hydroxide and 0.15 M hydrazine (in water or sodium chloride solution) solution was used as absorbent for the carbon dioxide.

Blank values, established for distilled water and all reagents except ninhydrin, were about 0.5 mm. lower by the "double equilibration" than by the "original technique."

Calculations—Factors for converting the differential pressures to millimoles were calculated from the data given by Van Slyke *et al.* (2) for 0.5 N sodium hydroxide and 2.0 N lactic acid, by Van Slyke and Folch (7) for similar solutions containing hydrazine, and by MacFadyen (3) for reagents containing 25 per cent sodium chloride with and without hydrazine. In the latter case it was assumed that hydrazine has a negligible effect on the solubility of carbon dioxide. The manometric chamber was calibrated according to the directions of Peters and Van Slyke (9) and all other volumetric apparatus by standard procedures.

In most of the experiments the reaction mixture was buffered at pH 2.5 and the carbon dioxide was measured with reagents made up in 25 per cent sodium chloride (3) (see Table I). A few amino acids were also analyzed with reaction mixtures buffered to pH 4.7 (see Table II). Reagents made up in distilled water only (2) were used to measure the carbon dioxide evolved in a few experiments to test the effect of salt (Table IV).

DISCUSSION

It was observed with most of the amino acids that the liberation of carbon dioxide was completed during the first few minutes of heating. In other cases heating was prolonged until the pressure readings were constant or dependable rate curves established from which the values for carbon dioxide at the standard times could be determined by interpolation.

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[5] If, before the upper cock is closed, nearly all of the gas is returned to the reaction flask by raising the mercury level nearly to the 2 ml. mark, the excess pressure is somewhat less than that obtained by the described procedure.

TABLE I

Manometric Analyses of Various Amino Acids by Means of Reaction with Ninhydrin at pH 2.5 with Salt-Saturated Reagents

Amino acid	Quantity used per determination	Original technique			Double equilibration technique			Hydrazine technique		
		No. of determinations	CO_2 per mole amino acid: Mean	CO_2 per mole amino acid: Mean deviation	No. of determinations	CO_2 per mole amino acid: Mean	CO_2 per mole amino acid: Mean deviation	No. of determinations	CO_2 per mole amino acid: Mean	CO_2 per mole amino acid: Mean deviation
	micromoles		*moles*	*per cent*		*moles*	*per cent*		*moles*	*per cent*
dl-Alanine . .	36	5	1 022	0.5	4	1.016	0.2	5	1.002	0.4
dl-Isoleucine . .	30	1	1 137		1	0.996		2	0 999	0 0
l(−)-Leucine . ..	32	5	1 145	0.8	1	1 023		3	1.001	0.3
dl-Norleucine	36	1	1 041		1	1 012		1	1.000	
dl-Norvaline	38	1	1.056		1	1.030		1	1.005	
dl-Valine	18–36	12	1 122	5.5	2	0 982*	0 7	4	1 001	0.2
Asparagine monohydrate (natural form)	35	1	1 011		1	1 008		3	1.006	0 4
dl-Aspartic acid	20	1	2 002		1	1.998		3	1.991	0.5
dl-Phenylalanine .	38	2	1.000	0 2	2	0.998	0.3	3	1.001	0.3
l(+)-Arginine monohydrochloride	24	5	1.005	0 6	2	1.001	0 5	1	1 004	
l(+)-Glutamic acid	35	1	0 996†		1	0.996		1	0.997	
l(−)-Histidine .	28	3	0.998	0.2	2	0 997	0 2	3	1 000	0.2
dl-Serine	40	1	1.001		1	1.001		2	0 998	0.1
dl-Threonine	39	1	0.996		1	0.996		2	0.995	0.2
l(−)-Tyrosine	38‡§	1	1,000		1	1.000		1	1.001	
dl-α-Aminocaprylic acid	32§	4	1 000	0.5	3	1.001	0 6	9	0 942	2.7
l(−)-Cysteine hydrochloride	30	1	0.982‖		1	0.981‖			0.96¶	
l(−)-Cystine	8§	1	2.049		1	2 045			2.09¶	
α-Amino-α-ethyl-*n*-butyric acid .	37								0.22¶	
Glycine . .	40	2	0.922	0 4	1	0.918		4	0.920	0.4
dl-Lysine dihydrochloride	20	1	1.070**		1	1 069**			1.09¶	
l(−)-Tryptophane	21								0.94¶	
dl-Methionine	30–36	20	1.011	0 4	14	1.011	0.5	6	1.009	0.4
l(−)-Proline	34		1.02¶			1.02¶			1.02¶	
l(−)-Hydroxyproline .	30	3	1.001	0.1	1	1.000		2	0.998	0.2

Unless otherwise indicated, the values entering into the means listed resulted from reaction times sufficiently long to yield steady values.

* Value low because of insufficient number of lowerings of the mercury.

† The glutamic acid was dissolved in 0.024 N hydrochloric acid, the solution was not neutralized, and the pH was about 2.1 after the addition of buffer.

‡ The volume of the aliquot was 2 ml. and 50 mg. of ninhydrin were used.

TABLE I—*Concluded*

§ The amino acid was dissolved in dilute hydrochloric acid. Each sample was brought to pH 2.5 with the requisite amount of buffer of pH 4.7 before the 50 mg. of buffer of pH 2.5 were added.

‖ The reaction time was 10 minutes.

¶ The quantity of carbon dioxide evolved in 7 minutes was estimated by interpolation of the curve given in Fig. 1.

** The reaction time was 5 minutes.

The data given in Table I indicate that the mean values obtained in the analysis of alanine, isoleucine, leucine, norleucine, and norvaline by the "original technique" are from about 2 to 13 per cent higher than the theoretical amounts. The errors with these amino acids are reduced in analyses by the "double equilibration technique," while they are negligible by the "hydrazine technique." The latter was the less satisfactory only in the case of α-aminocaprylic acid. This result may be explained by the formation and deposition on the surfaces of the manometric chamber of a coating of thin white leaves which impaired the absorption of the carbon dioxide. It seems probable that this product is heptylidenehydrazine which, according to Darapsky and Adamczewski (10) crystallizes as lustrous white leaflets melting at 133–136°. The 2.0 N lactic acid solution, added subsequently, dissolved this product.

The behavior of those amino acids observed to give abnormal values even by the hydrazine technique is represented by the curves given in Fig. 1. Although glycine is abnormal (Table I), no curve is shown, since the evolution of carbon dioxide stops in a few minutes (Table V). Although cysteine reacts more slowly than cystine and all but a few of the other amino acids, approximately the theoretical amount of carbon dioxide is finally liberated. The observation that more than the expected 2 moles of carbon dioxide is produced from cystine at pH 1, 2.5, and 4.7 differs from that of Van Slyke *et al.* (2) who obtained less than 2 moles of carbon dioxide at pH 2.5 and 4.7. It seems probable that the expected 1 mole of carbon dioxide would result from α-amino-α-ethyl-*n*-butyric acid if the reaction time were prolonged to 2 to 3 hours, although it may be assumed from the data in Table II that the original technique would give an erroneously high value. 1 mole of carbon dioxide is evolved rapidly from lysine and a 2nd mole at a slower rate, decreasing with increase in acidity of the medium. Carbon dioxide is liberated from tryptophane slowly at pH 2.5 in amounts approaching those obtained at pH 4.7 (Table II).

The relatively high values found with certain amino acids by the original technique indicate that some volatile substances in addition to carbon dioxide were present. Since there was marked improvement by the double equilibration technique, it seems probable that these substances were not acids. Because the odor of the fluid ejected from the manometric chamber was strongly aldehydic when the original or the double equilibration technique was used and was pleasant and slightly ammoniacal, but not aldehydic, when the hydrazine technique was employed, it was assumed that the volatile substances were aldehydes. According to Abderhalden (11) aldehydes are formed by the reaction of these amino acids with ninhydrin. Apparently, hydrazine acts to suppress the "aldehyde error" by transforming aldehydes to products of low volatility.

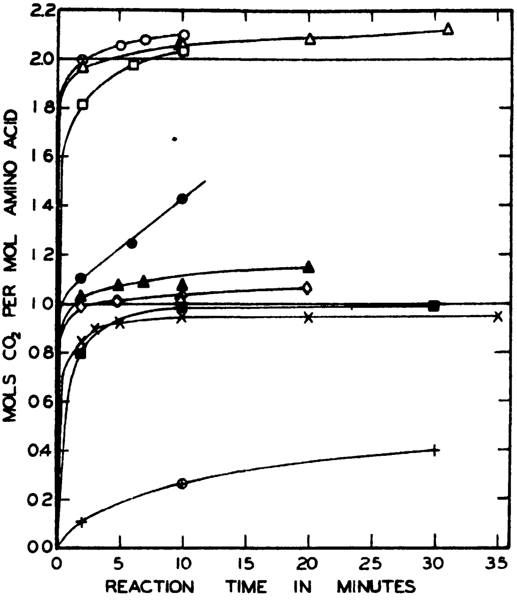

FIG. 1. The course of reaction of some amino acids with 50 mg. of ninhydrin in 1 ml. at 100°. Cystine at pH 2.5 ○, at pH 1.0 (0.1 N HCl) △, at pH 4.7 □, lysine at pH 4.7 ●, at pH 2.5 ▲, cysteine at pH 2.5 ■, tryptophane at pH 2.5 ×, α-amino-α-ethyl-*n*-butyric acid at pH 2.5 +, at pH 4.7 ⊕, proline at pH 2.5 ◇.

The following experiments were performed in order to determine the effect of pure aldehydes on the analysis by the ninhydrin method of carbon dioxide liberated from sodium carbonate. 1 ml. of 0.035 M sodium carbonate solution, 0.1 ml. of a 0.4 M solution of a pure aldehyde, and about 70 mg. of a buffer mixture of pH 2.5 contained in a glass cup of about 150 mg. capacity attached to a glass stilt were placed in a reaction flask. The flask was evacuated and sealed in the usual manner, the buffer was spilled by upsetting its container, the flask was placed for 5 minutes in a boiling water bath, and the liberated carbon dioxide was determined by the ninhydrin procedure, as previously described. It may be noted from the results of these experiments shown in Table III that the carbon dioxide found in the presence of *n*-butyraldehyde, propionaldehyde, and isobutyraldehyde by the original technique is in excess of the theoretical amount by about 1, 3, and 8 per cent, respectively. It is evident, also, that the carbon dioxide present and that found by the hydrazine technique agree, in each case, within negligible limits of experimental error.

TABLE II

Manometric Analyses of Various Amino Acids by Means of Reaction with Ninhydrin at pH 4.7 with Salt-Saturated Reagents

Amino acid	Quantity per determination	CO_2 found per mole amino acid analyzed		
		Original technique	Double equilibration technique	Hydrazine technique
	micromoles	*moles*	*moles*	*moles*
dl-Alanine	36			1.002
l(+)-Arginine monohydrochloride	24	1.006	1.006	1.005
Asparagine monohydrate (natural form)	35			1.050
l(−)-Cystine	8*			1.98†
α-Amino-α-ethyl-*n*-butyric acid	37	0.282‡	0.264‡	0.22†
Glycine	39	0.974	0.974	0.976
		0.979	0.978	0.972
l(−)-Leucine	32			1.000
dl-Lysine dihydrochloride	20			1.27†
l(−)-Tryptophane	21	0.987	0.988	0.983
dl-Methionine	30–36	1.010	1.010	1.014
		1.009	1.009	1.017
l(−)-Proline	34	1.011§	1.012	1.010
			1.007	1.009
l(−)-Hydroxyproline	30	1.016	1.006	
		0.996	0.996	
		1.005		

Unless otherwise indicated, the values entering into the listed means resulted from reaction times sufficiently long to yield steady values.

* The cystine was dissolved in 0.1 N hydrochloric acid. Immediately before the ninhydrin was added, 20 mg. of trisodium citrate dihydrate and 50 mg. of buffer of pH 4.7 were added to bring the pH to 4.7

† The quantity of carbon dioxide evolved in 6 minutes was estimated by interpolation of the curve given in Fig. 1.

‡ The reaction time was 10 minutes.

§ Mean of five determinations, with a mean deviation of 0.5 per cent.

Distilled water was used by Van Slyke *et al.* (2) as the solvent for the reagents employed for the analysis of amino acids, whereas MacFadyen (3) utilized 25 per cent sodium chloride solution for this purpose. The carbon dioxide could be determined more accurately by the latter method because of its relatively low solubility in salt solutions.

The present authors' experimental data (Tables I, II, and IV) strongly support the view that the "aldehyde error" is accentuated by the use of reagents containing sodium chloride. By the original technique values for leucine, isoleucine, and valine more than 4 per cent higher than the theoretical amount were obtained even with reagents prepared with distilled water. On the other hand, it was found, in harmony with the results of Van Slyke *et al.* (2), that the analysis of alanine with "distilled water reagents" gives the theoretical value by any of the three stipulated techniques.

It was considered desirable for practical purposes to determine the approximate time required for 99.9 per cent completion of the reaction of the amino acids with ninhydrin. The desired information was calculated by means of the first order law (2, 4)

TABLE III

Effect of Aldehydes on Manometric Analysis of Sodium Carbonate with Salt-Saturated Reagents

Aldehyde	CO_2 found per mole Na_2CO_3 present		
	Original technique	Double equilibration technique	Hydrazine technique
	moles	*moles*	*moles*
None	1.002	1.001	0.998
Propionaldehyde ..	1.027	1.007	1.006
n-Butyraldehyde	1.014	1.004	0.998
Isobutyraldehyde ...	1.081	1.006	1.003

$$t_2 = \frac{3t_1}{-\log(1 - x_1)}$$

Values of t_1 and x_1 for the amino acids which react normally were derived from analyses at pH 2.5 and 100° with 1 or 2 minute time intervals. Corresponding data for the amino acids which react abnormally were obtained by interpolation from the curves given in Fig. 1.

It is of practical interest to note (Table V) that, of all the amino acids studied, only α-amino-α-ethyl-*n*-butyric acid, cysteine, and valine require more than 7 minutes under the indicated conditions for the liberation of 99.9 per cent of the expected amount of carbon dioxide. Of some theoretical significance are the observations that the rate of evolution of carbon dioxide is increased by lengthening the carbon chain except in the case of glycine, as well as by introducing substituents except α-alkyl, β-alkyl, β-indole, and β-sulfhydryl groups. It appears, further, that the isomeric amino acids norleucine, leucine, isoleucine, and aminoethylbutyric acid react at rates decreasing in the order given.

The data obtained by means of the manometric ninhydrin method appear to be as reliable as other quantitative procedures for the determination of purity of amino acids. The evidence on which this conclusion is based is given in Table VI. In the case of glycine the values are sufficiently consistent to be dependable, although the results obtained from the analysis of 30 to 40 micromoles of this amino acid with 50 mg. of buffer and 50 mg. of ninhydrin in 1 ml. of water must be multiplied by 1.083 for reactions run at pH 2.5 and by 1.025 at pH 4.7. It was assumed in deriving these factors that the glycine preparation (Table VI) was analytically pure. However, Van Slyke *et al.* (2) found glycine to react quantitatively at pH 4.7.

Table IV

Manometric Analyses of Various Amino Acids by Means of Reaction with Ninhydrin at pH 2.5 with Salt-Free Reagents

Amino acid	Quantity per determination	Original technique			Double equilibration			Hydrazine technique		
		No. of determinations	CO$_2$ per mole amino acid		No. of determinations	CO$_2$ per mole amino acid		No. of determinations	CO$_2$ per mole amino acid	
			Mean	Mean deviation		Mean	Mean deviation		Mean	Mean deviation
	micromoles		*moles*	*per cent*		*moles*	*per cent*		*moles*	*per cent*
dl-Alanine	35	3	1.004	0.1	3	1.002	0.0	1	1.006	
dl-Aspartic acid	20	1	1.992		1	1.990				
l(+)-Glutamic acid	35*							1	0.993	
dl-Isoleucine	30	2	1.054	0.0	2	0.999	0.7			
l(−)-Leucine	32	2	1.046	0.4	2	1.005	0.8	2	1.000	0.1
dl-Norleucine	36	2	1.012	0.1	2	1.006	0.0			
dl-Norvaline	37	2	1.006	0.2	2	1.000	0.2			
dl-Valine	35	2	1.044	0.3	2	1.010	0.2			
dl-Methionine	31	3	1.004	0.3	2	1.006	0.3			

Unless otherwise indicated, the values entering into the listed means resulted from reaction times sufficiently long to yield steady values.

* The glutamic acid was dissolved in 0.024 N hydrochloric acid, the solution was not neutralized, and the pH was about 2.1 after the addition of buffer.

Lysine and cystine evolve additional carbon dioxide so rapidly under the experimental conditions that no accurate empirical rules could be devised. According to Van Slyke *et al.* (2) lysine may be determined most satisfactorily by the ninhydrin method if the reaction is conducted at pH 1.0. Cystine might best be determined by reduction followed by the ninhydrin analysis of the resulting cysteine.

At the present time analyses by the ninhydrin method of amino acids in biological materials have only comparative value. The accuracy of such data is unknown and could be determined only from a study of the interactions of the naturally occurring amino acids. Although such investigations were not made, a solution containing an approximately equimolar mixture of seventeen amino acids[6] found in protein hydrolysates was analyzed. The solution used for this purpose was prepared by dissolving the pure amino acids, each weighed to 0.03 mg., in 0.112 N hydrochloric acid, transferring the mixture to a 25 ml. volumetric flask, and diluting the solution in the flask to the mark. 1 ml. aliquots of this solution were analyzed with the results shown in Fig. 2.

TABLE V

Time Required for Decarboxylation of Various Amino Acids in Approximately 0.03 M Concentration by 5 Per Cent Solution of Ninhydrin at 100° and pH 2.5

Amino acid	Time	Amino acid	Time
	min		*min.*
l(−)-Hydroxyproline	1.2*	*dl*-α-Aminocaprylic acid	3.2‡
l(−)-Lysine	1.2†	*l*(−)-Leucine	3.4*
l(−)-Histidine	1.4*	*dl*-Threonine	3.7*
l(−)-Tyrosine	1.8*	*dl*-Serine	4.1*
Asparagine (natural)	2.2*	*dl*-Norvaline	4.2*
l(+)-Arginine	2.5*	*dl*-Methionine	4.5*
dl-Phenylalanine	2.7*	*dl*-Isoleucine	5.8*
l(−)-Cystine	2.9†	*l*(−)-Tryptophane	6.3§
Glycine	2.9*	*dl*-Alanine	6.6*
l(+)-Glutamic acid	2.9*	*dl*-Valine	8.3*
dl-Aspartic acid	3.0*	*l*(−)-Cysteine.	12.0‡
l(−)-Proline	3.2†	α-Amino-α-ethyl-*n*-butyric acid	100‖
dl-Norleucine	3.2*		

* The time required to reach 99.9 per cent of the 10 minute value was estimated by the first order law from a 1 or 2 minute value obtained by means of the hydrazine technique.

† The time required to reach the theoretical value was interpolated from the appropriate curve in Fig 1.

‡ The time required to reach 99.9 per cent of the theoretical value was estimated by the first order law from a 2 minute value obtained by means of the original technique.

§ The time required to reach 99.9 per cent of the asymptotic value was interpolated from the appropriate curve in Fig. 1.

‖ 90 per cent of the theoretical value was obtained in 90 minutes.

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[6] The following amino acids were employed in concentrations ranging from 1.55 to 3.96 mM per liter of solution: *dl*-alanine, *l*(+)-arginine monohydrochloride, *dl*-aspartic acid, *l*(−)-cystine, *l*(+)-glutamic acid, glycine, *l*(−)-histidine, *dl*-isoleucine, *l*(−)-leucine, *dl*-lysine dihydrochloride, *dl*-norleucine, *dl*-phenylalanine, *dl*-serine, *dl*-threonine, *l*(−)-tryptophane, *l*(−)-tyrosine, and *dl*-valine.

It is of interest that the values obtained by either the double equilibration or the hydrazine technique agree closely with those estimated by summation of the values interpolated from the experimental curves representing the rates at which carbon dioxide was liberated from each of the seventeen amino acids as measured by the hydrazine technique. Furthermore, approximately 1 mole of carbon dioxide per unit of reactive carboxyl was formed in 6 to 8 minutes at 100° and pH 2.5. It is evident that the error is about 2.5 per cent when the carbon dioxide is measured by the original technique with reagents made up in 25 per cent sodium chloride solution. Analyses run at pH 4.7 proved less satisfactory, the estimated value and that measured directly by the hydrazine technique being approximately 101 and 102.5 per cent, respectively, of the theoretical amount.

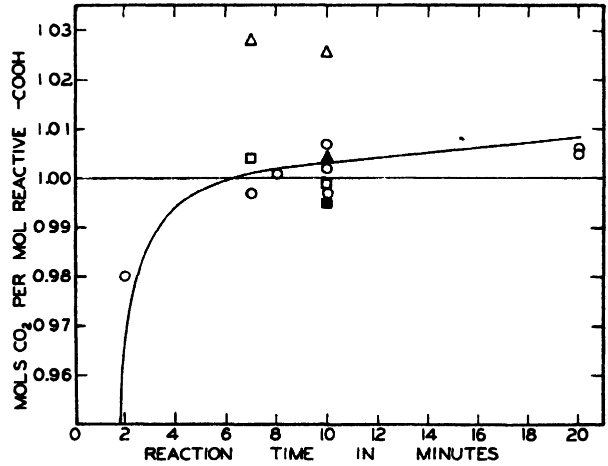

FIG. 2. The reaction of a mixed amino acid solution with ninhydrin at pH 2.5 and 100°. The curve is drawn through points calculated from the data previously obtained by study of the reactions of the individual amino acids with ninhydrin by the hydrazine technique. The open symbols represent measurements made with reagents containing 25 per cent sodium chloride, while the solid symbols represent measurements with salt-free reagents. Measurements made by the original technique are plotted as triangles, those by the double equilibration technique as squares, and those by the hydrazine technique as circles.

Theoretical Considerations

In its most general form the manometric method for measuring a gaseous substance in a 2-phase system may be described as follows:

Step 1—The gaseous phase is brought into equilibrium with an aqueous phase, the gas volume being G_1, the aqueous volume S, and the Ostwald distribution constants α'_{1A} for substance A, α'_{1B} for substance B, etc.

Step 2—The gas is compressed rapidly to a volume a at temperature t

TABLE VI

Estimated Per Cent Purity of Purified Amino Acids Based on Ninhydrin (Hydrazine Technique) and Other Methods of Analysis

Amino acid	Impurities			Purity	
	Moisture	Ash	Inorganic ions	Formol titration	Ninhydrin analysis
dl-α-Aminocaprylic acid			<0.04		100.1 ± 0.4[*]
dl-Alanine		<0.016			100.2 ± 0.3
l(+)-Arginine monohydrochloride[†]	<0 01	<0.02	<0 004		100.5 ± 0.4
Asparagine monohydrate (natural)[‡]	11.89		<0.04	98 4[‡]	99 4 ± 0.4[‡]
dl-Aspartic acid	<0.003		<0.004	100.0	99.6 ± 0.3
l(−)-Cysteine hydrochloride[§]			<0.004	98.6	99.3
l(−)-Cystine[‖]		<0.02	<0.004		101.2[¶]
α-Amino-α-ethyl-*n*-butyric acid	<0 05		<0.004	99.9	>90
l(+)-Glutamic acid			<0.004	99.6	99.5 ± 0.2
Glycine	<0 02	<0.026	<0.004	99.9	97.6 ± 0.1[**]
l(−)-Histidine			<0.04		99.9 ± 0 2
l(−)-Hydroxyproline			<0.15		99.8 ± 0 2
dl-Isoleucine	<0 08	<0.02	<0.004	100 1	99 8 ± 0 2
l(−)-Leucine[††]	<0 02			99.7	100.0 ± 0.2
dl-Lysine dihydrochloride[‡‡]					109[§§]
dl-Methionine	<0.01	<0.02	<0.004		101.1 ± 0 4
dl-Norleucine	<0 06	<0 02	<0 004	100.2	100 1 ± 0.5
dl-Norvaline	<0 01		<0 004	100 0	100 2 ± 0.3
dl-Phenylalanine[‖‖]			<0 04	100 0	100 0 ± 0 3
l(−)-Proline			<0 15		101 0 ± 0 3
dl-Serine[¶¶]	<0 03	<0 04	<0.004	100 3	99 9 ± 0 1
dl-Threonine			<0.15		99 6 ± 0.1
l(−)-Tryptophane[***]			<0.004		98 3 ± 0.2
l(−)-Tyrosine[†††]	<0 02	<0.02	<0 004	100.5	100 1 ± 0 1
dl-Valine	<0 01			100.0	100.1 ± 0 2

All of the listed amino acids with three exceptions were synthesized or isolated, purified, and analyzed in the authors' laboratory. *dl*-Threonine, *l*(−)-proline, and *l*(−)-hydroxyproline were Merck products, stated to contain less than 0.15 per cent inorganic ions and, respectively, 11.6 to 11.9, 12.0 to 12.3, and 10.6 to 10.8 per cent of nitrogen.

[*] By the "original technique."

[†] The purity was 100.5 per cent according to Volhard analysis of chloride.

[‡] The moisture analysis gave 99.0 per cent of the theoretical, the Kjeldahl analysis of nitrogen 99.5 per cent, the formol titration 101.6 per cent, and the ninhydrin analysis 100 6 per cent. On the assumption that the sole impurity is anhydrous aspartic acid the purity of the preparation in asparagine monohydrate corresponding to the above analytical data is, respectively, 99.0, 99.0, 98.4, and 99.4 per cent.

[§] The purity was 97.0 per cent according to Kjeldahl analysis of nitrogen.

[‖] The purity was 99.0 per cent according to Kjeldahl analysis of nitrogen and 103.3 per cent by sulfur analysis.

[¶] Value obtained in 8 minutes at pH 1.0. The value was 99.0 in 6 minutes at pH 4.7 and 104.5 in 7 minutes at pH 2.5.

TABLE VI—*Concluded*

** Value obtained at pH 4.7. The value at pH 2.5 was 92.3.

†† Contained less than 0.1 per cent methionine.

‡‡ The purity was 99.6 per cent according to Volhard analysis of chloride.

§§ Value obtained in 7 minutes at pH 2.5.

‖‖ The purity was 100.0 per cent according to Kjeldahl analysis of nitrogen and 100.6 per cent by Van Slyke manometric analysis of amino nitrogen.

¶¶ The purity was 100.2 per cent according to Van Slyke manometric analysis of amino nitrogen.

*** The purity was 97.2 per cent according to Kjeldahl analysis of nitrogen.

††† The purity was 99.6 per cent according to Kjeldahl analysis of nitrogen.

and pressure p. Reabsorption reduces the consequent supersaturation in the gaseous phase of the soluble components A, B, etc., by the factors j_{1A}, j_{1B}, etc.

Step 3—The volume of the aqueous phase is altered to S_2 and the Ostwald distribution constants are changed to α'_{2A}, α'_{2B}, etc. The system is equilibrated at the gas volume G_2.

Step 4—The gas is again compressed to the volume a at pressure p_2. Reabsorption reduces the supersaturation of each component by the factors j_{2A}, j_{2B}, etc.

Generalization of Van Slyke and Neill's (12) Equation 5, with a change in the form of the reabsorption correction, leads to the following expression for the contribution P_A of N_A millimoles of component A to the total differential pressure,

$$P_A = \frac{17{,}024(1 + 0.00384t)}{a}\left[\frac{1}{1 + \frac{S_1}{G_1}\alpha'_{1A}\left(1 + \frac{G_1 - a}{a} j_{1A}\right)} - \frac{1}{1 + \frac{S_2}{G_2}\alpha'_{2A}\left(1 + \frac{G_2 - a}{a} j_{2A}\right)}\right] N_A$$

Since, after the addition of alkali all of the carbon dioxide is in solution, *i.e.* $\alpha'_{2A} = \infty$ where A is CO_2, and since $G_2 = a$ in the original technique, this expression simplifies to Van Slyke and Neill's equation (with a changed reabsorption coefficient). Substitution of the value of Van Slyke *et al.* (2) for the distribution coefficient of carbon dioxide (at 25°) and the values of the other constants for the original technique leads to an expression for E_A, the relative effectiveness of A (any component) and carbon dioxide in contributing to the total differential pressure.

$$E_A = \frac{\frac{P_A}{N_A}}{\frac{P_{CO_2}}{N_{CO_2}}}\left[\frac{1}{1 + 0.064\alpha'_{1A}(1 + 22.5j_{1A})} - \frac{1}{1 + 1.75\alpha'_{2A}}\right] \times 1.064$$

Analogous expressions are readily derived for the relative effectiveness, E^D_A and E^S_A, in the respective double equilibration and submicromethods.

Although there are no α' values in the literature for aldehyde vapors, the relation $\alpha' = 16{,}700 - (\bar{S}/J)$, where J is the vapor pressure and $\bar{S}$ the molal solubility of a liquid or solid, holds at least in the neighborhood of saturation. Solubility values are available for a few aldehydes and vapor pressures may be estimated from boiling points. Some estimated values of α' in water for several aldehydes and the values of E_A, E^D_A, and E^S_A calculated from these and other values of α' on the assumption that $\alpha'_{(H_2O),A} = \alpha'_{1A} = \alpha'_{2A}$ are given in Table VII. It is evident from a consideration of these data and those given in Table IV that the values of E_A for $j_{1A} = 0.1$ are approximately in the same range of magnitude as the experimental "aldehyde errors." Insufficient data are available to explain the higher excess pressure observed with valine than with norvaline. The increase in E_A which accompanies a decrease in α' corresponds to the experimental finding that the "aldehyde error" is accentuated by the incorporation of sodium chloride in the reagents, thus decreasing their solvent power for non-polar substances. The relatively low values for E^S_A (based upon the greater supersaturation of the gas phase with a resultant greater reabsorption of aldehyde vapors) indicate that submicroanalyses performed with the three techniques would probably show less divergence than did the microanalyses we have reported.

TABLE VII

Relative Contribution of Different Substances to Total Differential Pressures of Particular 2-Phase Systems

Substance	α' in water	E_A $[j_1 = 0]$	E_A $[j_1 = 0.1]$	E^D_A $[j_1 = 0.1]$ $[j_2 = 0.1]$	E^D_A $[j_1 = 0.1]$ $[j_2 = 0.1]$	E^S_A $[j_1 = 0.1]$
Acetaldehyde	360	0.040	0.012	0.002	0.008	0.004
Propionaldehyde	178	0.090	0.023	0.004	0.016	0.008
Isobutyraldehyde	158	0.100	0.026	0.004	0.016	0.009
Butyraldehyde	124	0.124	0.033	0.006	0.020	0.012
Hypothetical substance	30	0.32	0.115	0.017	0.063	0.043
" "	10	0.56	0.27	0.033	0.129	0.117
CO_2 (if $\alpha'_2 = \alpha'_1$)	0.83	0.53	0.448	0.010	0.106	0.499
O_2	0.031	0.027	0.027	0.011	0.006	0.157

SUMMARY

It has been found that net pressures higher than the theoretical P_{CO_2} values result when some amino acids are analyzed by the manometric measurement of carbon dioxide liberated by the action of ninhydrin. The

formation of aldehydes which exert an appreciable partial pressure was considered to be the source of this error. It has been shown that this error may be suppressed by the use of reagents containing hydrazine.

A series of values has been presented denoting the behavior of various amino acids in reactions with ninhydrin at 100° at several pH levels by use of reagents with and without sodium chloride and hydrazine. It has been found that the theoretical values and those obtained with the hydrazine technique were in close agreement for eighteen of the twenty-four amino acids investigated.

It has been concluded from the evidence presented that certain pure amino acids may be analyzed individually and collectively with high accuracy by the hydrazine technique. The physical chemistry of the errors introduced by an extraneous volatile substance as a function of its properties and the conditions of the analysis is briefly treated.

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COMPOSITION OF CASEIN IN MILK

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(Received for publication, March 17, 1944)

The literature reveals numerous investigations (1) of the elementary composition of acid-precipitated casein but no recorded analyses of the percentage content of the elements of casein as it exists in the caseinate-phosphate complex in milk.

In this investigation a procedure for the separation of the casein complex from the other milk constituents by means of the Sharples supercentrifuge and the ultimate analysis of this complex is given. Also, experiments are described demonstrating the true chemical character of the calcium phosphate fraction of the complex. From the results of this investigation, the elemental composition of the calcium caseinate fraction and casein was then calculated.

Comparison of the results of the elemental composition of the casein obtained by this procedure with the values given in the literature for acid-precipitated casein should indicate whether the caseinate undergoes any change in elemental composition by acid precipitation other than substitution of hydrogen for calcium.

Preparation of Calcium Caseinate-Calcium Phosphate Complex

The bowl (approximately 300 ml. capacity) of the Sharples supercentrifuge was lined with a celluloid sheet and spun slowly until filled with distilled water. The speed of the bowl was then increased to 50,000 R.P.M. and 500 ml. of skim milk at 5° were run slowly into the bowl through a fine capillary, the rate of feed being such that about 20 minutes elapsed between the beginning and the end of the introduction of the skim milk. The skim milk was followed by 300 ml. of cold distilled water, with the speed of the bowl still at 50,000 R.P.M. The deposited caseinate was removed from the celluloid liner and ground sufficiently fine so that, when dispersed in 500 ml. of distilled water, it remained in suspension. This diluted suspension was then put through the supercentrifuge in a manner similar to that used in centrifuging the skim milk, by first filling the lined bowl with distilled water and finally by running in 300 ml. of additional water after the introduction of the emulsion. The washing of the complex was then repeated in the same manner. The twice washed deposit, which contained about 65 per cent of moisture, was allowed to dry in the air on the celluloid sheet, the temperature being maintained at 20° so as to minimize fermentation. The dried material was then ground to pass a No. 60 sieve and stored in a securely stoppered glass bottle to prevent loss or gain in moisture. Samples for study were removed as desired. This material gave a negative test for lactose and chlorides, indicating freedom from serum components. However, ether extracted 0.015 per cent of fat; a correction was accordingly applied in the calculations of the results of the elementary composition.

Methods of Analysis

Nitrogen was determined by the semimicro-Kjeldahl method, with about 30 mg. of material and digestion for 8 hours as recommended by Chibnall, Rees, and Williams (2), in a Parnass-Wagner (3) digestion apparatus. The value reported is the average of six determinations. Calcium determinations were made on the ash obtained by incinerating in a muffle furnace and analyzing by the official macro volumetric permanganate method ((4) p. 127) and total phosphorus by the official gravimetric method ((4) p. 21). Inorganic phosphorus was obtained from the trichloroacetic acid filtrate and determined by the strychnine gravimetric method of Embden and Fetter (5). Values of four determinations did not differ by over 0.003 per cent. Organic phosphorus was calculated by determining the difference between total and inorganic phosphorus. Moisture was determined by heating in a vacuum oven to constant weight, while the temperature was not permitted to rise above 105°. Carbon and hydrogen determinations were made by the procedure outlined by Clark (6), with a semimicro combustion furnace described by this author. The official sodium peroxide fusion method ((4) p. 132) was used for the determination of sulfur.

Results of Analysis

The results of the ultimate analysis of the calcium caseinate-calcium phosphate complex are shown in the first and second lines of Table I.

Before the elementary composition of the calcium caseinate and of the casein could be calculated from the analysis of the complex, it was first necessary to establish the chemical formula of the calcium phosphate fraction so that the calcium could be correctly allocated between the protein and the inorganic phosphate.

Chemical Nature of Inorganic Phosphate Associated with Milk Caseinate

If the value 0.958 per cent obtained for the inorganic phosphorus is calculated as the tribasic phosphate, the complex will contain 4.80 per cent $Ca_3P_2O_8$ and 95.20 per cent calcium caseinate; or if it is calculated as the dibasic phosphate, the complex will contain 4.21 per cent $CaHPO_4$ and 95.79 per cent calcium caseinate. As indicated in the reactions given below, addition of neutral potassium oxalate to the complex composed of the caseinate and the dibasic salt will not appreciably change the pH, while, on the other hand, if $Ca_3P_2O_8$ is present in the complex, the mixture will become more alkaline due to the formation of K_3PO_4.

$$CaHPO_4 + K_2C_2O_4 \rightarrow CaC_2O_4 + K_2HPO_4 \tag{1}$$

$$Ca_3P_2O_8 + 3K_2C_2O_4 \rightarrow 3CaC_2O_4 + 2K_3PO_4 \tag{2}$$

Consequently, increase in alkalinity will only be manifested if the tribasic salt is present in the complex.

The action of neutral oxalate on calcium phosphate has been studied by Van Slyke and Bosworth (7) and by Pyne (8) in their investigations of the nature of the insoluble inorganic phosphate in milk. They added the oxalate to milk and serum, however, and not to the isolated complex.

TABLE I

Per Cent Composition of Calcium Caseinate-Calcium Phosphate Complex

	Moisture	Calcium	Phosphorus		Nitrogen	Carbon	Hydrogen	Sulfur	Oxygen, by difference
			Organic	Inorganic					
Complex .	10.06	2.680	0.667	0.861	13.13	44.76	5.923	0.653	
" dry basis*. .		2.982	0.742	0.958	14.60	49.78	6.587	0.726	
Calcium caseinate†.		1.180	0.780		15.34	52.29	6.919	0.762	22.73
Casein .			0.789		15.51	52.88	6.997	0.771	23.05

* Also ether extract-free.

† 95.20 per cent of complex.

To determine, by the application of this reaction, which compound, the dibasic or tribasic phosphate, is associated with the caseinate, the following experiment was undertaken.

Two 1 gm. samples of the complex were transferred to 200 ml. glass-stoppered bottles. To the first were added 50 ml. of distilled water and 2 ml. of toluene, and to the second 30 ml. of distilled water, 20 ml. of neutral 4 per cent potassium oxalate, and 2 ml. of toluene. Each mixture was then brought to a pH of 9.08 by adding, in small amounts and with occasional shaking, a total of 5.09 ml. of 0.05 N sodium hydroxide to the first and 0.48 ml. of 0.05 N hydrochloric acid to the second over a period of 3 days. The final pH of 9.08 was obtained only after holding the suspension for 20 hours following the final addition of the alkali or acid. Preliminary titrations indicated that at a pH of 9.08 the buffer effect was small and that any small addition of the standard alkali or acid yielded a distinct change in pH. The sum of the alkali and acid added represented 0.2785 ml. of normal solution. This value, calculated to represent tricalcium phosphate, gave a concentration of 4.81 per cent $Ca_3P_2O_8$ in the complex, which is in good agreement with the value of 4.80 per cent $Ca_3P_2O_8$ calculated from the analysis. If the salt had been the dibasic phosphate, then the addition of neutral oxalate would not have changed the pH perceptibly from that of the sample without oxalate. We have isolated complexes that varied slightly in relative proportion of caseinate and phosphate from the values given in this paper. Variations that we found in percentage content of the two fractions of the complex are explained as due to different techniques used in washing the complexes, since difference in procedure might alter the relative proportions of the two components. These variations give credence to the assertion by some investigators (7, 9, 10) that the calcium phosphate is not bound chemically to the calcium caseinate fraction.

To obtain further evidence of the character of the inorganic phosphate fraction, so that the proper proportion of the calcium can be assigned to the protein fraction, a comparison was made of the pH of a water dispersion of the complex with pH values of a series of dispersions obtained from artificially prepared calcium caseinates of known calcium contents. The calcium caseinates were prepared from acid-washed and water-washed grain curd casein. The grain curd casein was obtained from fresh skim milk by precipitation with dilute hydrochloric acid, then washed thoroughly with acidulated distilled water, followed with distilled water, and was finally subjected to a pressure of 200 pounds for 20 hours to remove as much wash water as possible without actually drying the curd. The moist curd was then dispersed in distilled water and treated with highly diluted calcium hydroxide. During the period of reaction of the casein and calcium hydroxide, the mixture was agitated violently. Throughout, the reaction was kept below pH 8, and finally adjusted to the desired pH by the addition of more casein. The dispersions were then filtered through cheese-cloth and the calcium caseinate was freed from the mother liquor by passing the dispersions through the supercentrifuge, redispersing in distilled water, and recentrifuging. The moist caseinates were then air-dried, ground to pass a No. 60 sieve, and analyzed for calcium and inorganic phosphorus. The results of the analysis are given in Table II.

The pH values of 3 per cent water suspensions of these four calcium caseinates are plotted against their calcium percentages in Fig. 1. The pH determinations were made with the Beckman glass electrode. For purposes of comparison that part of the Palmer and Richardson (11) titration curve within similar pH range is included. These authors constructed their curve from data obtained by titrating potentiometrically 1 per cent casein-water dispersions with standard calcium hydroxide solution.

If the inorganic phosphate exists as a tribasic salt, the calcium in excess of the requirement of this salt would give a caseinate of only 1.18 per cent calcium and, according to the graph, give a pH of 6.40, a value very near to that of the milk itself.

Lack of precise agreement between Palmer and Richardson's curve and ours is undoubtedly due to the marked differences in the methods by which the curves were obtained. These authors used a highly purified casein, following, in its preparation, the procedure outlined by Van Slyke (12). That differences in base-binding capacity of caseins prepared by different methods do exist has been pointed out by Cohn and Berggren (13).

TABLE II

Analysis on Dry Basis of Calcium Caseinate Prepared from Grain Curd Casein

Experiment No.	pH of 3 per cent dispersion	Phosphorus, inorganic	Calcium, total	Calcium, corrected for Ca in $Ca_3P_2O_8$
		per cent	*per cent*	*per cent*
1	6.20	0.020	1.074	1.035
2	6.59	0.036	1.362	1.292
3	6.99	0.081	1.728	1.571
4	7.40	0.088	1.858	1.680

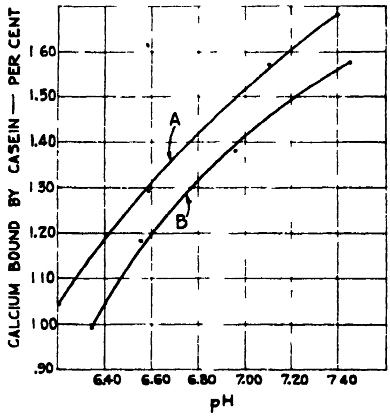

FIG. 1. Relationship between pH and calcium percentage of calcium caseinate. Curve A, 3 per cent calcium caseinate emulsion; Curve B, 1 per cent calcium caseinate emulsion, "Palmer and Richardson."

Thus, having established the existence of only $Ca_3P_2O_8$, and not $CaHPO_4$, in the complex, we then calculated the ultimate analysis of the calcium phosphate fraction and casein from the analytical data of the complex. The results are incorporated in Table I (third and fourth lines).

The value of 1.18 per cent calcium is somewhat higher than the values given in the literature. Söldner (10) reports 1.07 to 1.11 per cent, Lehmann (14) 1.04 per cent, and Ling (15) 1.00 per cent. We have isolated samples of the complex with a calcium content as low as 1.02 per cent calcium after correcting the total calcium found by the calcium bound to the inorganic phosphorus calculated as $Ca_3P_2O_8$. These variations in calcium bound by the protein are evidence of differences in milks. Small differences could arise also from experimental errors of the methods of analysis; *i.e.*, errors within the limits of accuracy of the methods.

In the literature there is no analysis of the elementary composition of casein obtained by our procedure, and that the results check, as a whole, surprisingly well with analyses of casein obtained by acid precipitation is interesting evidence that the changes in physical character through the action of acid are not accompanied by a pronounced alteration in its percentage elemental composition. However, the fact that the percentages we found for sulfur and phosphorus are somewhat higher than those obtained for casein repeatedly dissolved by alkali and precipitated by acid indicates that such treatment removes a portion of these elements from casein.

SUMMARY

A new procedure for obtaining the elementary composition of casein is described. The technique is to isolate the calcium caseinate-calcium phosphate complex from milk by means of the supercentrifuge, analyze the complex, and from the results calculate the percentage content of the elements of the casein. Such a procedure avoids separation and alteration of the casein by chemical means and, in consequence, prevents loss of phosphorus and sulfur. Otherwise, the percentage composition of casein as determined by this procedure does not differ appreciably from values given in the literature.

Increase in alkalinity of the complex by the addition of neutral potassium oxalate indicates the presence of tricalcium phosphate rather than dicalcium phosphate in the complex.

On this basis the complex was found to contain 4.80 per cent $Ca_3P_2O_8$ and 95.20 per cent calcium caseinate.

The calcium caseinate fraction of the complex was found to contain 1.18 per cent calcium.

A series of calcium caseinates was prepared from grain curd casein and the percentages of calcium were plotted against the pH of 3 per cent dispersions prepared from them. The curve indicates that a caseinate of 1.18 per cent calcium content would yield a pH of 6.40, which is only 0.07 pH more acid than the pH of the milk from which the complex was obtained.

Note—Since the preparation of this paper, an article by de Kadt and van Minnen (16) has come to our attention. These authors described the partial fractionation of the complex by means of the Sharples supercentrifuge and found that the inorganic phosphate exists as the tribasic calcium phosphate and that the ratio between the caseinate and inorganic phosphate is not always constant. They also found that slightly more phosphate is centrifuged out with the first fraction than with the following fractions.

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THE INHIBITION OF CATALYZED OXIDATIONS BY HEMINS*

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(Received for publication, April 13, 1944)

In earlier studies (1) it was demonstrated that the oxygen uptake of brain tissue brei is greatly enhanced by the addition of iron salts, particularly an iron complex formed with the dye *o*-phenanthroline ($C_{12}H_8N_2$), without corresponding liberation of CO_2. A comparison of this effect on breis of various other body organs revealed the anomalous behavior of the spleen, which, itself unaffected, actually inhibits catalysis of brain oxidation by the dye. The inhibitory agent in spleen was identified first with the red blood cell, then with hemoglobin. The mechanism of this inhibition is here further explored, with fatty acid or a phospholipid fraction of brain as substrate. These more purified materials were substituted for brain tissue, since the oxidations were shown to involve principally the phospholipids of brain.

Method

Throughout these studies the Warburg technique was employed to follow oxygen uptake by the substrates, with added catalyst or hemin compound or both. Linoleic acid, obtained from a commercial source, was twice distilled *in vacuo*, and 0.01 cc. was suspended in each vessel in 1.6 cc. of M/15 phosphate buffer at pH 7.3. The phospholipid was prepared from cattle brain in the following manner. 300 gm. of brain were first extracted with four 300 cc. portions of acetone, the acetone was filtered off (Buchner funnel), and the residue dried *in vacuo*. This residue was now extracted four times with 200 cc. portions of hot alcohol-ether (3:1 mixture), the solids were filtered off and discarded, and the alcohol-ether distilled *in vacuo*. The phospholipid residue was dissolved in about 200 cc. of ether, filtered, and then precipitated from solution with 3 volumes of acetone. The resultant product was quickly dried with the aid of a vacuum oil pump and kept under nitrogen. It was homogenized in phosphate buffer and used in 2 to 40 mg. quantities, more commonly the latter. The catalyst here is ferrous-*o*-phenanthroline, 6×10^{-4} M final concentration. The hemin compounds tested include hemoglobin purified according to Welker,[1] catalase prepared from liver after Sumner and Dounce (3), and cytochrome *c* obtained from heart (Keilin and Hartree (4)). The hemoglobin was 8 to 16×10^{-6} M (initial solution 0.5 to 1.0 per cent), while the cytochrome, 4×10^{-5} M (0.7 per cent initially), contained a comparable amount of hemin. Catalase, 3×10^{-9} M to 3×10^{-7} M, had at best 0.01 the hemin of the other two. All experiments were carried out at 37° in air.

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* Presented before the American Society of Biological Chemists at Boston (*Federation Proc.*, **1**, pt. 2, 134 (1942)).

[1] Welker, W., personal communication. See also Hawk and Bergeim (2).

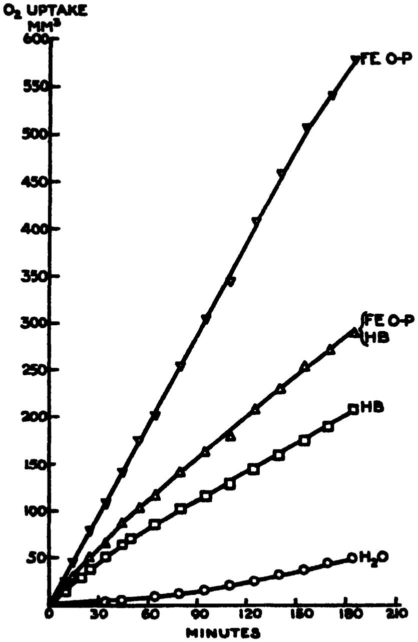

FIG. 1. Oxygen uptake of linoleic acid. ▽, with 0.0062 M ferrous-*o*-phenanthroline added; △, with 0.0062 M ferrous-*o*-phenanthroline and 1 per cent hemoglobin added; □, with 1 per cent Hb added; ○, H_2O control.

Results

The essential fatty acid, linoleic ($C_{17}H_{31}COOH$), autoxidizes at a slow, though measurable rate, after an induction period of roughly half an hour. The iron-dye catalyst, in 6×10^{-3} M concentration, increases this rate about 10-fold; 1 per cent hemoglobin halves this acceleration but itself increases the "basal" oxidation 4-fold. These results are presented graphically in Fig. 1. Hemoglobin catalysis of unsaturated fatty acid oxidation, though much studied, is incompletely understood. Haurowitz *et al.* (5) suggest that the reaction proceeds through an intermediate peroxide stage, and that hemoglobin is destroyed in the process. Assuming that catalase would attack the organic peroxide involved here, which it might not, this was added to the system in an attempt to keep the reaction from going to the right. No such action was demonstrable, but it was incidentally noted that catalase, a hemin, up to 3×10^{-7} M has no catalytic action. The third hemin compound tested, cytochrome *c*, increases linoleic oxidation approximately 50 per cent as much as does the equivalent amount of hemin as hemoglobin.

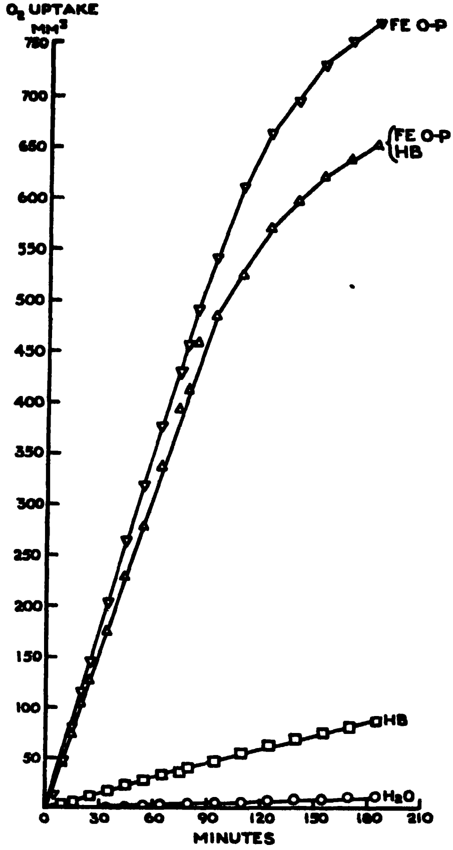

FIG. 2. Oxygen uptake of phospholipid (40 mg. per flask). ▽, with 0.0062 M ferrous-*o*-phenanthroline added; △, with 0.0062 M ferrous-*o*-phenanthroline and 1 per cent Hb added; □, with 1 per cent Hb added; ○, H_2O control.

Cyanide does not inhibit hemin catalysis of oleic acid oxidation (Kuhn and Meyer (6)), nor hemoglobin catalysis of linoleic acid oxidation (Robinson (7)), but it does diminish the effect of ferrous-*o*-phenanthroline on oxygen uptake of brain brei (1). If cyanide would inhibit the iron-dye catalysis of fatty acid oxidation, then, barring direct combination between the toxic agent and the dye complex, hemoglobin (or hemin) and iron-phenanthroline might be acting by two different routes, one sensitive, the other insensitive to cyanide. Careful experiments revealed only temporary inhibition and suggest that hemoglobin and the dye may be competing in the same way for the substrate; hemoglobin, by virtue of its lower catalytic rate, may thus reduce the net catalytic effect.

Hemoglobin did not increase the oxygen usage of brain brei in the early experiments. Brain tissue is high in phospholipid content (about 5 per cent; see Bloor (8)), but the suspension used was quite dilute, and perhaps for this reason did not respond. Hemoglobin catalysis similarly is not seen with small amounts of a more purified phospholipid preparation. With 1 per cent hemoglobin and increasing amounts of phospholipid, catalysis is evident; a 7-fold increase in oxygen uptake results when 1 per cent hemoglobin is added to 40 mg. of phospholipid (Fig. 2). The iron-dye effect is a dramatic 700-fold increase, and this is somewhat depressed by the hemin compound. From Fig. 3 it is readily apparent that cytochrome *c*, which elevates linoleic oxygen uptake to 2.5 times the control value, has no effect on phospholipid.

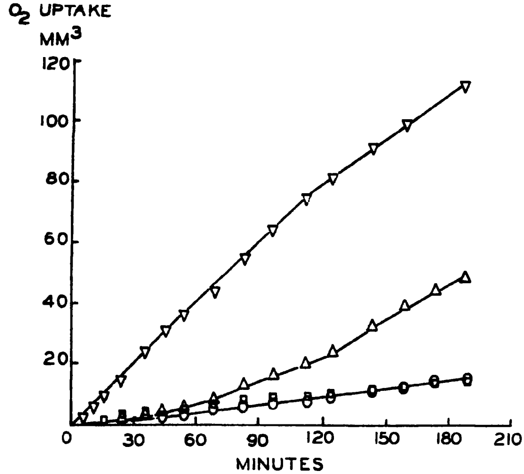

FIG. 3. Effect of cytochrome *c* on oxygen uptake of linoleic acid with cytochrome, ▽, without cytochrome, △; phospholipid with cytochrome, □, without cytochrome, ○. Cytochrome concentration 0.7 per cent, approximately equivalent to 1 per cent Hb in hemin content.

SUMMARY

Hemoglobin increases the oxidation of essential fatty acid and of phospholipid, and would probably do the same for a sufficiently concentrated brain suspension. Cytochrome *c* is effective only with linoleic acid as substrate, and then but half as effective as hemoglobin. Catalase was noncatalytic in the quantities utilized, perhaps because the hemin concentration was too low. Ferrous-*o*-phenanthroline markedly enhances oxidation of all substrates used, and hemoglobin depresses these accelerations. Phospholipids have been designated as a source of intracellular reserve energy for bull spermatozoa (Lardy and Phillips (9)). If they serve a similar purpose in brain and other tissues (and Gerard and Tupikova (10) have shown they are oxidized away in nerve), then hemins, while acting as mild catalysts for deriving energy from these reserves, might at the same time preserve them from too rapid destruction by more active catalysts. The cyanide data presented indicate an identity of mechanism for hemoglobin and ferrous-phenanthroline action. If hemoglobin adsorbed on the substrate partially displaces the more active catalyst, the total catalytic rate would be less with both than with only the more active catalyst present.

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THE CONFIGURATION OF VALYLVALINE IN GRAMICIDIN

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One of the curious problems posed by the polypeptides produced by *Bacillus brevis* is the presence of both *d*- and *l*-valine (1–3) in gramicidin hydrolysates. Does an enzyme of this organism fail to differentiate between the two optical antipodes? Or is *d*(−)-valine incorporated specifically into one position in gramicidin and *l*(+)-valine into a different one, just as *d*(+)-leucine is incorporated into gramicidin (4, 5) and *l*(−)-leucine into tyrocidine (6) by this organism? The separation of the dipeptide valylvaline (7) from gramicidin hydrolysates presents an opportunity to answer this question. If one assumes that each of the two positions of valine in the valylvaline molecule represents a single specific position in the gramicidin molecule, then determination of the configuration of the valylvaline should show whether or not *Bacillus brevis*, in elaborating gramicidin, differentiates between the two enantiomorphic valines.

There are possible four isomeric valylvalines and two racemic modifications;[1] namely,

(1) *d*(−)-Valyl-*d*(−)-valine
l(+)-Valyl-*l*(+)-valine (Abderhalden and Vlassopoulos (8))

(2) *d*(−)-Valyl-*l*(+)-valine (Fischer and Scheibler (9))
l(+)-Valyl-*d*(−)-valine

The preparation isolated from gramicidin might be any one of the four isomers (provided that its optical rotation is small), or either racemic form, or any other mixture of the isomers. In this investigation, comparison with synthetic valylvaline derivatives showed that the isolated dipeptide was the optically inactive *dl* mixture, *d*(−)-valyl-*d*(−)-valine + *l*(+)-valyl-*l*(+)-valine. Evidence was obtained indicating that appreciable quantities of the other two isomers were not present in the hydrolysates.

First the four isomeric benzoylvalylvalines, and also their ethyl esters, were synthesized. Appreciable optical activity was shown by the benzoylvalylvalines as sodium salts; therefore, the product separated from gramicidin could not be any single isomer, since it showed little if any optical activity (7). Equal quantities of enantiomorphic pairs of the benzoylvalylvalines, and also of their ethyl esters, were crystallized together to obtain the optically inactive *dl* mixtures. The two optically inactive *dl* mixtures of benzoylvalylvalines failed to melt sharply, whereas the two racemic esters showed no significant depression of melting points when mixed. Hence the product isolated from gramicidin could not be identified by comparison with these preparations.

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[1] In this paper, the term racemic modification is used interchangeably with the term optically inactive *dl* mixture. No implication of interaction between the members of the enantiomorphic pairs is intended.

The initial failure to demonstrate the identity of the crystals isolated from gramicidin with any of the synthetic benzoylvalylvalines led to a reinvestigation of the former. In addition to yielding after acid hydrolysis a major part of its nitrogen as *dl*-valine derivatives, half of the nitrogen was represented by *l*(+)-valine, as shown by assay with *Lactobacillus arabinosus* (10). Thus the earlier conclusion as to the composition of the product isolated was strengthened.

Because valylvaline appears to be unusually stable to acid hydrolysis, a test was made to see whether the partial hydrolysis of benzoylvalylvaline yielded any valylvaline. A degree of acid hydrolysis releasing 17 per cent of the nitrogen of benzoyl-*l*(+)-valyl-*l*(+)-valine as amino nitrogen did not yield detectable amounts of the dipeptide.

In the selection of other derivatives by which differentiation of the two racemic forms might be accomplished, the following was considered. When two amino acids of like configuration are in contiguity in a peptide chain, the side chains are held to project from opposite sides of the peptide chain, whereas when a *d*-amino acid occurs next to an *l*-amino acid, the side chains are close together. Except in the case of 2- and 3-carbon amino acids, these side chains occupy a large portion of the available space (11). It seemed probable that attachment of a large group at the carboxyl end by the ester link would exaggerate the steric congestion of racemic modification (2) compared with modification (1), and that this would be reflected by a large difference in melting point. Thus the ethyl ester of benzoyl-*d*(−)-valyl-*l*(+)-valine melted at a temperature 7° lower than that of benzoyl-*d*(−)-valyl-*d*(−)-valine. An attempt to prepare the *p*-nitrobenzyl ester of racemic form (2) resulted in the separation of an oil. The *p*-phenylphenacyl esters, however, were prepared with practically quantitative yields, and their melting points showed a separation permitting sharp differentiation between the two forms. The ester of the inactive mixture, benzoyl-*d*(−)-valyl-*d*(−)-valine + benzoyl-*l*(+)-valyl-*l*(+)-valine melted at 201°, whereas the ester of the other racemic modification melted at 141–142°.

Four preparations of benzoylvalylvaline from gramicidin were studied, isolated after different periods of hydrolysis and by procedures designed to avoid or detect any loss of valylvaline isomers. These preparations were converted to *p*-phenylphenacyl esters. All melted sharply at 201°, showed no depression of melting point when mixed with the ester of racemic form (1) but exhibited strong depression when mixed with the ester of form (2).

Hence the isolated preparations all consisted of the optically inactive *dl* mixture, benzoyl-*d*(−)-valyl-*d*(−)-valine and benzoyl-*l*(+)-valyl-*l*(+)-valine.

Admixture of 5 per cent of the ester of form (2) with the ester of form (1) caused appreciable depression of the melting temperature. When equal quantities of the four isomeric benzoylvalylvalines were mixed and carried through the isolation and esterification procedure together, a product was obtained which melted gradually from 140–180°. These results indicate that fractionation during isolation probably does not account for the failure to recover benzoyl-*d*(−)-valyl-*l*(+)-valine and benzoyl-*l*(+)-valyl-*d*(−)-valine. The same conclusion may be drawn from the fact that substantially all of the peptide nitrogen appearing in the fraction which formed water-soluble copper salts was isolated as valylvaline when the period of hydrolysis of the gramicidin was 13 hours.

Studies of the comparative rates of release of amino nitrogen and of the various amino acids of gramicidin have been made in collaboration with Hegsted, with microbiological assay methods for the amino acids. These results will be published subsequently. They show an extremely rapid release of *l*(+)-valine during the first few minutes of hydrolysis of gramicidin, followed by a very slow release. After 2 hours and after 6 hours, the fractions of valine that were unavailable to the test organism closely approximated the amounts that have been isolated as valylvaline. Thus it appeared that during the first few minutes of hydrolysis the reoccurred a rapid release of valine and of valylvaline, and that subsequently valine arose largely from the slow cleavage of valylvaline. The correspondence of the amounts of unreleased valine found by bioassay with the amounts of valylvaline isolated demonstrated that the isolation of the dipeptide was nearly quantitative and that no considerable quantities of *d*(−)-valyl-*l*(+)-valine or *l*(+)-valyl-*d*(−)-valine could be present.

EXPERIMENTAL

Isolation of Valylvaline from Gramicidin—194 mg. of gramicidin were hydrolyzed by hydrochloric acid (7) for 13 hours. At this point, the "peptide nitrogen" (the increase which could be produced in amino nitrogen by 12 hours of additional hydrolysis in a sealed tube at 130°) was 3.0 per cent of the gramicidin nitrogen taken. The solution was boiled for an additional 15 minutes under aeration in the presence of 0.1 ml. of benzaldehyde to render indole-containing compounds insoluble. Chloride was removed with silver oxide and the copper salts were prepared as previously described and extracted with two 3 ml. portions of water. The extract was dried and extracted with three 3 ml. portions of absolute ethanol. The alcoholic extract was dried and dissolved in water, and copper was removed with hydrogen sulfide. The solution contained 0.96 mg. of amino nitrogen and 0.49 mg. of peptide nitrogen. After the solution was evaporated to dryness, the solutes were dissolved in 0.2 ml. of absolute ethanol and 0.2 ml. of acetone was added. After 2 days, the mother liquor was removed from the crystals of crude valine, evaporated to dryness, the residue redissolved in ethanol, and again precipitated by acetone. Finally, by gradual addition of 5 parts of diethyl ether, valylvaline was crystallized as needles containing 6.5 per cent amino nitrogen (calculated for valylvaline, 6.48 per cent). The yield was 9 mg., thus including most of the peptide nitrogen. The identification was made by benzoylation as described below.

Preparations of Benzoylvalylvaline from Gramicidin. Preparation G345—Mother liquors from two preparations of benzoylvalylvaline studied previously were concentrated *in vacuo* and an additional small crop obtained, melting at 216–218°.[2]

Preparation G475—200 mg. of gramicidin were hydrolyzed by acid as usual for 6 hours. This hydrolysis converted 67 per cent of the total nitrogen to amino nitrogen and left 13 per cent of the nitrogen as "peptide nitrogen." The substances forming copper salts insoluble in 30 ml. of water were discarded. The resultant solution, which contained all of the peptide nitrogen, was treated with benzoyl chloride at a volume of 2 ml., an excess of sodium bicarbonate being used to maintain alkalinity during benzoylation. The solution was acidified and, after 2 weeks, the precipitate was removed and crystallized twice from acetone. Recovery from the second crystallization was nearly quantitative. The yield of valylvaline represented 4.5 per cent of the nitrogen of the gramicidin used. The preparaton melted at 216°. These modifications in the isolation method were made to secure the maximum yield and to reduce the possibility of fractionation of isomers.

Preparation G496 was obtained from a 2 hour hydrolysate of 100 mg. of gramicidin. During the last 10 minutes of hydrolysis, tryptophane-containing compounds were destroyed by aerating in the presence of benzaldehyde. The water-soluble, ethanol-soluble, copper salts were separated as described above. This fraction contained as amino nitrogen 11.6 per cent of the nitrogen of the gramicidin taken and 8.69 per cent as peptide nitrogen. The benzoyl derivative was prepared and separated as usual. The yield represented 4.8 per cent of the nitrogen of the gramicidin taken.

Preparation G490 was obtained by benzoylating the valylvaline isolated above. It melted at 218°.

Determination of l(+)-Valine in These Fractions—Weighed portions of

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[2] All preparations described were dried *in vacuo* at 100° over P_2O_5 with the exception of the acid chlorides which were dried at 55°. The melting points recorded have been corrected for the emergent thermometer stem.

Preparations G475 and G345 were subjected to hydrolysis in acetic acid-hydrochloric acid solution (1 volume of acetic acid plus 2 volumes of 6 N hydrochloric acid) at 100° and 110° in sealed tubes for varying periods of time. The hydrolysates were taken to dryness, washed with benzene, and taken up in water. Upon aliquots of the resultant solution amino nitrogen was determined by the Van Slyke technique and *l*(+)-valine by the method of Hegsted (10) with *Lactobacillus arabinosus*.[3] Table I shows that, within the error of the biological assay, half of the amino nitrogen was present in the form of *l*(+)-valine.

Benzoyl-d(−)-valyl Chloride and Benzoyl-l(+)-valyl Chloride—Benzoyl-*d*(−)-valine and benzoyl-*l*(+)-valine were prepared as described for benzoyl-*dl*-valine by Slimmer (12) from *d*(−)-valine and *l*(+)-valine,[4] respectively, and benzoyl chloride. The preparations melted at 127° (13). They were converted to the acid chlorides as follows: 300 mg. of benzoylvaline were dissolved in 2 ml. of freshly distilled acetyl chloride. At 0°, 315 mg. of phosphorus pentachloride were added and the mixture was shaken for 5 minutes at 0°, then for 30 minutes at room temperature. 8 ml. of anhydrous petroleum ether were added and the solution was left at 0° to complete the crystallization. The needles were centrifuged down and washed three times with petroleum ether. Both preparations melted gradually from 95–105°. Titration with standard sodium hydroxide solution indicated 2 equivalents of acid in 242 and 243 gm. respectively. Benzoylvalyl chloride should require 239.6 gm.

TABLE I

l(+)-Valine Released by Hydrolysis of Benzoylvalylvaline Preparations from Gramicidin

Preparation No.	Hydrolysis*	Amino N	*l*(+)-Valine
		mg. atoms per l.	*mM per l.*
G345	18 hrs. at 100°	2.98	1.5
G475	22 " " 100°	2.40	1.3
G475	42 " " 110°	2.01	1.1

* Hydrolysis was by a solution of 1 volume of acetic acid and 2 volumes of 6 N hydrochloric acid.

Benzoylvalylvaline Ethyl Esters—The ethyl esters of *d*(−)- and *l*(+)-valine were prepared as ethereal solutions, as described for leucine by

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[3] The biological assays of *l*(+)-valine were made by Dr. D. M. Hegsted.

[4] The *d*(−)-valine was generously supplied by Dr. William C. Rose of the University of Illinois, and the *l*(+)-valine by Dr. Edwin J. Cohn and Dr. John Ferry of the Department of Physical Chemistry, Harvard Medical School. Determination of specific rotations confirmed the purity of the two isomers.

Fischer (14), and the solutions were dried over calcium oxide. Nitrogen analyses on the ethereal solutions indicated yields of about 80 per cent.

154 mg. (0.65 mM) of benzoyl-*d*(−)-valyl chloride were added to a solution of an equivalent amount of *d*(−)-valine ethyl ester and 1 mM of anhydrous pyridine in 20 ml. of ether, and dissolved by shaking a few seconds. In the course of 16 hours at room temperature, the solution became filled with long needles of benzoylvalylvaline ethyl ester. Without removal of the needles the solution was washed three times with 5 ml. portions of dilute hydrochloric acid and four times with 5 ml. portions of 5 per cent sodium bicarbonate and twice with water to remove pyridinium chloride, valine ethyl ester, and benzoylvaline. The solid phase disappeared during the washing. The solution was finally taken to complete dryness *in vacuo*. The white product was crystallized from boiling ligroin as fine needles with a yield of 70 per cent. After two or three crystallizations from 50 per cent ethanol, the compound melted at 166°.

$C_{19}H_{28}O_3N_2$. Calculated N 8.04, mol wt. 348 2
Found. " 7.97, neutralization equivalent (by saponification) 352

In the same way, benzoyl-*d*(−)-valyl chloride was allowed to react with *l*(+)-valine ethyl ester, and benzoyl-*l*(+)-valyl chloride in separate experiments with *d*(−)- and *l*(+)-valine ethyl esters. The composition of the products was checked by nitrogen analyses and determination of neutralization equivalents. The melting points were as follows: benzoyl-*d*(−)-valyl-*l*(+)-valine ethyl ester 159°, benzoyl-*l*(+)-valyl-*d*(−)-valine ethyl ester 160°, benzoyl-*l*(+)-valyl-*l*(+)-valine ethyl ester 166°. All were slender diamond-shaped plates. The yields were between 65 and 70 per cent.

Crystallization together of equal amounts of benzoyl-*d*(−)-valyl-*d*(−)-valine ethyl ester and benzoyl-*l*(+)-valyl-*l*(+)-valine ethyl ester from 50 per cent alcohol yielded crystals melting at 153°. Similarly, the crystals obtained by crystallizing together the other two esters melted at 152°. No appreciable depression of melting point resulted when these racemic modifications were mixed.

Benzoylvalylvalines. By Saponification of Esters—To 110 mg. of benzoylvalylvaline ethyl ester were added 1.5 ml. of 0.25 N alcoholic sodium hydroxide and 1 ml. of 95 per cent alcohol. The solution resulting was left at room temperature overnight, then concentrated *in vacuo* to about 0.3 ml., and 0.3 ml. of 2 N hydrochloric acid added. The solution was evaporated *in vacuo* nearly to dryness, and the precipitate rubbed up with 2 ml. of water, then washed twice more with water. The precipitate was dried and crystallized three times from dry methyl acetate. Large monoclinic prisms were formed from all four esters. These were somewhat hydroscopic, moderately soluble in acetone and ethyl acetate, very soluble in alcohol and in aqueous alcohol, and insoluble in petroleum ether and in water. The yields from the saponification were about 50 per cent. The reason for this incomplete recovery was not investigated, since the procedure described below was found more satisfactory for preparing the benzoylvalylvalines.

By Action of Benzoylvalyl Chloride on Valine in Alkaline Solution—100 mg. of benzoylvalyl chloride were added to the equivalent quantity, 59 mg., of valine in 1.0 ml. of molar sodium carbonate solution, the mixture being cooled in tap water and agitated until the solids dissolved. The solution was acidified with hydrochloric acid and, after standing overnight, the precipitate was removed, washed with water, dried, and recrystallized three times from methyl acetate. The yields were between 58 and 65 per cent of the theoretical. The products so obtained were shown to be identical with those prepared above by melting point determinations. Analyses, specific rotations, and melting points are shown in Table II.

TABLE II

Analysis and Properties of Benzoylvalylvalines

Compound	M.p	N content*	Neutralization equivalent†	$[\alpha]^{25}_{\text{Hg}}$ as sodium salts ±1°
	°C	*per cent*		*degrees*
Benzoyl-*d*(−)-valyl-*d*(−)-valine	193–195	8.7	322	+21
Benzoyl-*l*(+)-valyl-*l*(+)-valine	193–195	8.8	325	−23
Benzoyl-*d*(−)-valyl-*l*(+)-valine	192–194	8.8	320	−17
Benzoyl-*l*(+)-valyl-*d*(−)-valine	192–194	8.7	323	+15

* Calculated N content, 8.75 per cent.

† Calculated, 320.2.

Optically Inactive dl Mixtures—5 mg. of benzoyl-*d*(−)-valyl-*d*(−)-valine and 5 mg. of benzoyl-*l*(+)-valyl-*l*(+)-valine were mixed and recrystallized three times from acetone. In the same way the other pair of isomers was mixed and crystallized together. The products obtained did not show satisfactory melting point behavior, softening at 195° and melting gradually to become clear at 210–215°. Some crystals were observed to melt at the lower, others at the higher temperatures, suggesting crystallization in at least two forms. Slow crystallization over a 2 week period yielded similar results.

p-Nitrobenzyl Esters—5.9 mg. each of benzoyl-*d*(−)-valyl-*l*(+)-valine and benzoyl-*l*(+)-valyl-*d*-(−)-valine were mixed and treated as their sodium salts in 63 per cent alcohol with 7.8 mg. of *p*-nitrobenzyl bromide, as described by Reid (15). Several attempts to crystallize the product were unsuccessful, yielding only a colorless oil.

Phenylphenacyl Esters (16)—Commercial *p*-phenylphenacyl bromide was purified by decolorization with charcoal in alcohol solution and by crystallization from alcohol. 4.9 mg. each of benzoyl-*d*(−)-valyl-*l*(+)-valine and its optical enantiomorph were dissolved in 0.2 ml. of ethanol. 0.03 milliequivalent of sodium hydroxide in 0.1 ml. of water was then added, followed by 8 mg. of *p*-phenylphenacyl bromide. The solution was held at its boiling point for 60 minutes. The product began to crystallize during this period. After cooling for a few hours, the crystals were removed, washed with water, and then extracted three times with 3 ml. quantities of warm petroleum ether to remove residual phenylphenacyl bromide. The residue was crystallized twice from 0.2 ml. portions of absolute ethanol.

$C_{31}H_{34}O_5N_2$. Calculated, N 5.44; found, 5.4; needles melting at 141–142°

In the same way, equal parts of benzoyl-*d*(−)-valyl-*d*(−)-valine and its enantiomorph were mixed and converted to the racemic *p*-phenylphenacyl ester. This formed needles melting sharply at 201°. A mixture of the two racemic forms melted gradually between 120–140°. In both cases, the yields were about 90 per cent. Both racemic forms were insoluble in cold ethanol.

3 mg. portions of the four preparations of benzoylvalylvaline derived from gramicidin hydrolysates described above were converted to phenylphenacyl esters in the manner described. All four melted sharply at 201° and showed no depression when mixed with the latter of the above esters, but strong depression resulted in admixture with the former racemic modification.

2.0 mg. of each of the four synthetic benzoylvalylvalines were dissolved together in 4 ml. of 0.1 N sodium hydroxide. The solution was acidified and the precipitate was crystallized twice from acetone, and converted to the phenylphenacyl ester, which was crystallized twice from ethanol. The product melted gradually from 120–175°, revealing no extensive changes in the composition of the mixture of isomers under the conditions employed in preparing the derivative of valylvaline from gramicidin.

Is Valylvaline Formed by Acid Hydrolysis of Benzoylvalylvaline?—5.6 mg. of benzoyl-*l*(+)-valyl-*l*(+)-valine were hydrolyzed in a mixture of 0.2 ml. of acetic acid and 0.4 ml. of 6 N hydrochloric acid at 100° for 30 minutes. The solution was evaporated to dryness, and the residue dissolved in 0.75 ml. of water and extracted with ether to remove benzoyl derivatives. The ether extracted 0.40 mg. of nitrogen; the aqueous solution contained 0.085 mg. of nitrogen, all in the form of amino nitrogen. Thus, no significant amount of valylvaline had resulted.

DISCUSSION

The finding that gramicidin hydrolysates contain similar quantities of *d*-valyl-*d*-valine and *l*-valyl-*l*-valine and little if any *d*-valyl-*l*-valine or *l*-valyl-*d*-valine demonstrates that neither racemic valine nor racemic valylvaline has arisen by racemization during hydrolysis and isolation.

Two alternatives should be considered: two valylvaline fragments may occur at different points in the gramidicin molecule, *d*-valyl-*d*-valine at one point and *l*-valyl-*l*-valine at another; or these two fragments may occur at the same point in different gramicidin molecules. The difficulty involved in the first alternative is that the two valylvalines must both be in special environments permitting their release at similar and rapid rates. The second alternative requires that there be two isomeric gramicidin molecules differing as to which enantiomorphic valylvaline they contain.

Whether or not *Bacillus brevis* contains an enzyme which fails to differentiate between *d*-valine and *l*-valine in joining valine to a dissimilar amino acid in gramicidin cannot yet be answered. The organism does, however, appear to join together only valines of like configuration. This arrangement of valine residues is the one which results in less crowding of side chains, since it places the two side chains on opposite sides of the peptide chain. It is also evident that less crowding of side chains will result from inserting two *d*-amino acid residues successively among *l*-amino acid residues in a peptide chain than will result from inserting them at isolated points.

SUMMARY

Valylvaline separated as the free dipeptide and as the benzoyl derivative under various conditions from gramicidin hydrolysates has been found to be the pure optically inactive *dl* form, $d(-)$-valyl-$d(-)$-valine and $l(+)$-valyl-$l(+)$-valine, by showing the phenylphenacyl ester of the benzoyl derivative to be identical with the synthetic racemic form. The findings indicate that no substantial quantities of the other two possible isomers were present in the hydrolysates. Hence optically inactive valylvaline could not have arisen by racemization during hydrolysis or isolation. These conclusions suggest that *Bacillus brevis* joins together in gramicidin only valines of like configuration.

Several derivatives of valine have been described.

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THE ISOLATION AND PROPERTIES OF SOME NATURALLY OCCURRING OCTADECENOIC (OLEIC) ACIDS

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(Received for publication, April 29, 1944)

Oleic acid (cis-9,10-octadecenoic) has been said to be the most widely occurring of the fatty acids. The best preparations of this acid have been made by Bertram (1), Raymond (2), Brown and Shinowara (3), Skellon (4), Smith (5), Wheeler and Riemenschneider (6), and Foreman (7). Olive, tea seed, and almond oils have served as the sources of these several preparations, but olive oil has been usually selected as the raw material, because of the relatively simple nature of its component fatty acids, and because of its high oleic acid content (75 to 80 per cent). The synthetic oleic acid of Noller and Bannerot (8) was believed to be identical with the natural acid.

In spite of the fact that, theoretically, there can exist sixteen isomeric octadecenoic acids, depending on the position of the double bond, only two or three of these, in addition to oleic acid, have been found to be naturally occurring. Petroselenic acid (6,7-octadecenoic) was found in parsley seed oil by Vongerichten and Kohler (9) and in ivy seed oil by Palazzo and Tamburelli (10). Vaccenic acid (11,12-octadecenoic) was identified in whale oil by means of its dihydroxy derivative by Moore (11) and by Armstrong and Hilditch (12). Bertram (13) claimed to have isolated vaccenic acid from beef fat and to have identified it. This same acid has been reported in butter fat, mutton fat, and lard in very small amounts. The presence of a 10,11-octadecenoic acid in pork liver lipids was suggested by Channon, Irving, and Smith (14).

Although several of the earlier investigators claimed high purity for their products, the preparation of pure oleic acid was almost impossible by the methods employed. This was due to the occurrence of the acid along with saturated acids on the one hand, and more unsaturated acids, such as linoleic, on the other. The methods of separation from both types of extraneous material were far from quantitative, many of the methods being based on differences in solubility of divalent and other metal salts. Furthermore, efficient stills were not available, so that the separations were complicated by the presence of C_{16} and C_{20} acids, both saturated and unsaturated.

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* Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of The Ohio State University.

The isolation of oleic acid has been greatly simplified by the method of low temperature crystallization, first used as a major procedure by Brown and Shinowara (3). The method has been modified and improved by Smith (5), Hartsuch (15), Wheeler and Riemenschneider (6), and, in this laboratory, by Foreman (7).

The essential details of Foreman's method include careful fractionation of the methyl esters of olive oil through an electrically heated and packed column, which results in a product containing only C_{18} esters, removal of methyl linoleate by several crystallizations from acetone or methyl alcohol at −60°, and removal of methyl stearate by cooling this product to −25° to −30° in methyl alcohol solution. The resultant methyl oleate upon saponification and one or two further crystallizations of the acid gave oleic acid which was 99.8 per cent pure.

In the present report we have attempted the isolation of oleic acid from several animal and seed fats and oils. The procedure employed was essentially that used by Foreman with such modifications as seemed necessary to adapt the method to the component C_{18} esters of the individual fat under investigation. One of our objectives was to demonstrate that the resultant product was identical with or different from the oleic acid derived from olive oil, which we have taken as a standard of purity. When a product of different properties has been obtained through this use of a simple physical procedure, it was the natural conclusion that the octadecenoic acid of the fat in question was in fact a mixture of octadecenoic acids. For example, it is generally accepted that the C_{18} acids of lard and of olive oil are stearic, oleic, and linoleic acids, the previous claim of the presence of vaccenic acid in lard being usually ignored. Crystallization of these acids, or their esters, should yield the same oleic acid in either case. However, as will be shown later, the oleic acid resulting from lard, while essentially pure as evaluated from the iodine number, melts considerably lower than the oleic acid from olive oil and gives upon non-disruptive oxidation a dihydroxystearic acid (or acids) which appears to be a mixture. This does not necessarily confirm the presence of vaccenic acid in lard, but it does support the likelihood that other octadecenoic acids than oleic do exist in this fat.

As will be seen from the results which follow, the oleic acid preparations from several animal fats, with the exception of chicken fat, appear to be mixtures. The preparations from olive, peanut, corn, cottonseed, and linseed oils appear to be oleic acid, while, again, those from rape-seed and soy bean oils show evidence of being mixtures.

EXPERIMENTAL

Several of the seed oils used in this work were specimens purchased on the market. Corn oil was furnished through the courtesy of the Corn

Products Refining Company, Argo, Illinois. Lard and beef tallow were obtained from Professor L. E. Kunkle of the Department of Animal Husbandry, the tallow being principally kidney fat. The chicken fat was obtained for us from white Leghorn hens by Professor E. L. Dakan of the Department of Poultry Husbandry. The C_{18} fraction of human fat was supplied by Cramer in connection with an investigation he was carrying out on that fat (16). Pork liver lipids were supplied by Dr. David Klein of The Wilson Laboratories, and adrenal phosphatides by Dr. Oliver Kamm of Parke, Davis and Company. The methyl esters of the adrenal phosphatides were by-products in the preparation of arachidonic acid by Mowry, Brode, and Brown (17).

TABLE I

Analytical Data of C_{18} Esters of Various Fats and Oils

Source	Mol. wt.		I No. of esters
	Esters	Acids	
Lard	295.5	281.5	86.5
Beef tallow	294.1	280.1	58.0
Adrenal phosphatides	297.5	283.5	103.5
Pork liver lipids	294.3	280.3	83.3
Chicken fat	297.4	283.4	102.4
Human fat	296.7	282.7	87.9
Linseed oil	294.5	280.5	197.5
Peanut "	292.8	278.8	118.9
Soy bean oil	293.6	279.6	116.0
Cottonseed oil	292.3	278.3	143.4

Description of Procedure and Results

Oleic acid was prepared from each of the preceding fats and oils by the following general procedure.

Preparation of Methyl Esters—The methyl esters were prepared by the usual method of direct alcoholysis. They were distilled before being subjected to more careful fractionation.

Fractional Distillation—The methyl esters were distilled very carefully through an electrically heated column packed with glass helices (25 mm. in diameter and 90 cm. long). In the usual practice in this laboratory it has been possible to separate mixtures of methyl esters into main fractions containing almost entirely (more than 95 per cent) single carbon series. Table I describes the C_{18} esters of the several fats and oils under investigation.

The molecular weights in Table I support the conclusion that the fractions in question are essentially C_{18} esters.

Isolation of Methyl Oleate—The crystallization procedure was applied to the C_{18} esters of each of the several fats and oils. Methyl stearate was removed by cooling either in methyl alcohol or acetone solution (2 to 3 per cent concentration) to −25° to −30°. The filtrate containing the oleate and more unsaturated esters was then cooled to −60°, the resulting crystals of methyl oleate being repeatedly crystallized until the iodine numbers of the crystal and filtrate fractions indicated complete removal of linoleate. In most of the fats studied linoleic acid was the only acid present which was more unsaturated than oleic. In others, however, linolenic and other highly unsaturated acids were present.

Owing to limitations of space it is impossible to give the details of the numerous crystallizations which were employed in the isolation of substantially pure methyl oleate from each of the fats. Details will be given later of the procedures employed with lard and linseed oils. Details of the crystallization of the C_{18} esters of human fat have been described by Cramer and Brown (16). It will suffice to note here the iodine numbers of the several methyl oleates which were isolated in this investigation and from which were prepared the several oleic acids described later. The iodine numbers and purities, calculated from the iodine numbers, are summarized in Table II.

TABLE II

Iodine Number and Purity of Methyl Oleate Preparations

Source	Iodine No.*	Per cent purity
Lard	83.40	97.4
Beef tallow	84.48	98.7
Adrenal phosphatides	83.67	97.7
Liver lipids	84.60	98.8
Chicken fat	85.92	99.6
Human fat	84.27	98.5
Linseed oil	86.64	99.0
Peanut "	85.67	100.0
Soy bean oil	85.39	99.8
Cottonseed oil	84.35	98.5

* Theory, 85.6.

Preparation and Purification of Oleic Acids—The methyl oleate preparations were saponified by refluxing for ½ hour with an excess of alcoholic potassium hydroxide, the alcohol removed under reduced pressure, and the oleic acid set free by addition of hydrochloric acid. The product was carefully distilled and analyzed; the analytical constants of the several preparations are given in Table III.

Since the analytical data on several of the products of the original series of crystallizations indicated purities which were not considered satisfactory, these were subjected to additional crystallizations to remove possible

TABLE III

Analytical Data on Oleic Acids

Source	Times recrystallized	n^{20}	M.p.	I No	Thiocyanogen No.	Purity, from I No	Analysis, thiocyanogen No			Mol wt.
							Oleic	Saturated	Linoleic	
			°C.				*per cent*	*per cent*	*per cent*	
Animal fats										
Lard	0	1.4602	12.3–12.6	87.74	87.85	97.6	97.7	2.3		
Beef tallow	0	1.4599	10.4–10.7	89.70	87.20	99.2	93.6	3.5	2.9	
Adrenal phosphatides	0	1.4598	11.4–11.5	86.89	84.24	96.6	90.1	6.8	3.1	
Liver lipids	0	1.4601	12.4–12.6	87.70	84.70	97.5	90.2	6.3	3.5	
Chicken fat	0	1.4602	13.0–13.4	88.87	88.40	98.8	97.5	1.9	0.6	
Human fat	0	1.4599	12.4–12.7	88.76	87.81	98.7	96.3	2.6	1.1	
Seed fats										
Linseed oil	0	1.4601	13.1–13.3	88.65	88.41	98.6	97.9	1.6	0.3	
Peanut "	0	1.4601	13.2–13.5	90.10	89.80	99.7	99.3	0.3	0.4	
Soy bean oil	0	1.4598	12.1–12.4	87.02	85.15	96.8	92.1	5.7	2.2	
Cottonseed oil	0	1.4601	13.1–13.2	89.09	88.00	99.1	94.7	3.9	1.4	
Olive oil*	0	1.4600	13.3–13.5	89.98	89.90	100.0	99.8		0.2	
Corn "†	0	1.4601	13.0–13.3	87.05	87.15	96.8				
Rape-seed oil‡	0	1.4598	12.5–12.7	87.26	85.76	97.0				
Recrystallized acids										
Lard	2		12.4–12.5	88.81	88.09	98.8	96.9	2.2	0.9	280.0
Beef tallow	7		11.3–11.5	87.93	87.58	97.8	97.8	1.8	0.4	282.7
Adrenal phosphatides	7		12.2–12.5	88.0	86.00	97.9	92.9	4.7	2.4	280.7
Human fat	6		12.9–13.0	88.97	87.18	98.9	94.5	3.4	2.1	282.2
Linseed oil	2		13.3–13.5	90.06	89.13	99.8	97.8	1.1	1.1	280.5
Soy bean oil	2		12.5–12.7	86.30	85.92	96.0	94.9	4.6	0.5	
Rape-seed oil‡	2		12.3–12.5	88.27	85.05	98.2	90.3	5.9	3.8	

* This was the best oleic acid preparation made by H. D. Foreman.

† Prepared by J. F. Frankel.

‡ Prepared by H. D. Foreman and S. L. Gans and further crystallized by one of us (R. C. M.).

traces of methyl esters which might have escaped saponification and of acids more unsaturated than oleic. Data on these recrystallized acids are also shown in Table III.

Discussion of Analytical Data on Oleic Acid Preparations

Refractive Index—The n^{20} of the several preparations in Table III ranged from 1.4598 to 1.4602, which values compare favorably with those previously reported: 1.4597 (5), 1.4599 (6), 1.4600 (7).

Melting Point—Previously reported melting points are, 13.0° (1, 3), 13.0–13.2° (6), 13.36° (5), and 13.3–13.5° (7). With the exception of the specimens from soy bean and rape-seed oils, the observed melting points of the seed fats fall very close to the above values. On the other hand, the specimens of animal origin, with the exception of chicken fat, melted significantly lower. The lowest melting point was shown by the oleic acid from beef tallow, which melted 3° low and 2° low even after seven additional crystallizations. These were made to remove possible traces of unsaponified methyl ester and linoleic acid, and, also, are likely to have removed some of the isomeric octadecenoic acids. These low melting points are discussed further in connection with remarks on the probable purity of the specimens.

Purity of Specimens—Purities have been evaluated from the iodine number and by calculation from the iodine-thiocyanogen equations of Matthews, Brode, and Brown (18). We are convinced that purities by the former method are more accurate than by the latter for two reasons. First, all of our preparations were crystallized to complete removal of acids more unsaturated than oleic acid, with the possible exceptions of peanut and linseed oils. Second, a slight error in the thiocyanogen determination tends to exaggerate the amount of impurity. In this laboratory on a number of occasions we have obtained low thiocyanogen values (1 to 2 units) on preparations of monoethylenic acids which we are certain do not contain diethylenic material. It is conceivable that certain of the isomeric octadecenoic acids do not add thiocyanogen quantitatively. These low thiocyanogen values, when used in the calculation, tend to indicate too high a percentage of both linoleic and saturated acids. While there is no doubt that some of our preparations contained as much as 2 to 3 per cent of saturated acid, we feel certain they did not contain up to 6 per cent as indicated in the oleic acids from adrenal phosphatides and liver lipids.

The purities of the unrecrystallized specimens in Table III as evaluated from the iodine number range from 96.6 to 99.2 per cent in the animal fats and 96.8 to 100 per cent in the seed fats. The principal impurity as indicated by iodine number is due to saturated acids, such as stearic and palmitic. In view of the fact that the starting material in each case was essentially C_{18} esters, it is not likely the contaminant was palmitic acid. Smith (5) reported only slight lowering of the melting point of oleic acid by stearic acid. The presence of 3.55 per cent stearic acid gave maximum lowering to 13.1°. Assuming complete absence of diethylenic acids, therefore, the low melting points of the oleic acid preparations from lard and especially from beef tallow and adrenal phosphatides do not appear to be caused by the presence of palmitic and stearic acids.

Melting Points of Dihydroxy Acids—Upon non-disruptive oxidation of oleic acid, there results an almost quantitative yield of a dihydroxystearic acid melting at 131–132°, one of the most characteristic reactions of oleic acid. A number of the preparations in Table III were oxidized by the procedure of Lapworth and Mottram (19). The resulting dihydroxy acids were crystallized once from alcohol, and the melting points taken. In a number of instances the mixed melting point with the known dihydroxy acid of olive oil oleic acid was observed. These results are summarized in Table IV.

TABLE IV

Melting Points of Dihydroxystearic Acids

Description of oleic acid	M.p., uncorrected	Mixed m.p. with olive oil dihydroxy acid
	°C.	°C.
Olive oil	130 -131	
Lard	124 -126	122–123
" (2)*	128.5–129.5	130–131
Beef tallow (7)	125.7	125–126.5
Adrenal phosphatides (7)	123 -124.5	125–127
Liver lipids	129 -130.5	131–132
Human fat	127.5–128.5	129–130
" " (6)	129 -131	131–132
Chicken fat	129 -131	129–131.5
Linseed oil (2)	130 -132	
Soy bean oil (2)	127.5–130	128–130
Cottonseed oil	130 -131.5	127–129
Rape-seed " (2)	130 -131	127–129

* The figures in parentheses refer to the number of times the oleic acid was recrystallized before the derivative was prepared.

The dihydroxy acids from olive oil, liver lipids, chicken fat, linseed oil, cottonseed oil, and rape-seed oil showed normal melting points. Those from lard, beef tallow, human fat, soy bean oil, and adrenal phosphatides melted appreciably lower, the minimum melting point being observed with adrenal phosphatides. The mixed melting point behavior was not entirely consistent but in general supported the conclusion that in a number of these preparations dihydroxy acids were being obtained which are quite different from the oxidation product to be expected from ordinary oleic acid. These low melting dihydroxy acids may or may not be identical with those obtained by Moore (11) and by Armstrong and Hilditch (12). Moore obtained a dihydroxystearic acid melting at 124–125° from South Sea whale oil. The product of Armstrong and Hilditch, also from whale oil, melted at 125–126°. On the basis of disruptive oxidation, these latter investigators concluded that this oil contained small amounts of the 11,12-octadecenoic acid.

Recrystallization of Acids—In a number of instances in which the evidence pointed to the presence of isomeric octadecenoic acids in the oleic acid, it was subjected to repeated recrystallization as noted in Table III, and the several filtrates from these crystallizations were further studied. As already reported by Cramer and Brown (16) in the case of human fat oleic acid, the consecutive filtrate fractions, amounting to 0.8 to 3.0 gm., melted at 1°, 2°, 8°, 9°, 10°, and 11°, respectively, indicated that these filtrates were concentrates of the isomeric acid. Several of these were combined (second and third) and after two crystallizations from petroleum ether at −65° yielded a product which melted at 5.7–6.0° and gave a dihydroxy acid melting at 122–123.5° (mixed melting point 125–127°). Similar low melting points were observed on the oleic acid in the filtrates from the repeated crystallization of the oleic acid from tallow, lard, adrenal phosphatides, soy bean oil, and liver lipids, and on the dihydroxy acids prepared from these filtrates. We believe that these results still further confirm our previous suggestions that the oleic acids from most of these animal lipids are mixtures of octadecenoic acids, of which ordinary oleic acid is the principal component.

To summarize, we have prepared oleic acids from twelve fats and oils of both animal and seed origin by a procedure which involved first, isolation of the C_{18} methyl esters of these fats by distillation through a very efficient column, separation of these C_{18} esters by repeated low temperature crystallization into methyl oleate, stearate, and more unsaturated esters, and conversion of the oleate into oleic acid by the usual method. Many of these oleic acids have been further repeatedly crystallized to remove possible impurities. In general we have found that four of the seed fat oleic acids (olive, cottonseed, corn, linseed) and one from an animal source (chicken fat) are practically pure oleic (9,10-octadecenoic) acid. The products obtained from soy bean and rape-seed oils, and those from lard, beef tallow, adrenal phosphatides, pork liver lipids, and human fat gave evidence of being mixtures of one or more octadecenoic acids, of which oleic acid is the principal component.

Isolation of Oleic Acid from Lard

The C_{18} esters of lard were repeatedly crystallized according to the procedure described in Chart I.

The methyl oleate from Chart I, upon saponification, gave an oleic acid with an iodine number of 86.65. This was crystallized as shown in Chart II. Since we were unable to account for the low iodine (and thiocyanogen) number in C_{10} of Chart II, this fraction was still further crystallized. See Chart III.

There is no evidence in these recrystallizations of the presence of linoleic or of palmitic acid. Yet it seems practically impossible here to remove the material which is giving an iodine number about 1 unit low. The evidences in Charts I to III for isomeric octadecenoic acids are in line with the points previously presented, more especially the melting points of the acid and of the several filtrate fractions. There is a reasonable possibility of the presence in this lard oleic acid of octadecenoic acids which do not give the theoretical iodine or thiocyanogen numbers. Since, however, they have not been identified other than as noted in the filtrates above, by actual separation or by disruptive oxidation, this conclusion is not beyond question.

CHART I

Fractional Crystallization of C_{18} Esters of Lard

225 gm. esters
I No. 86.4, thiocyanogen No. 64.4, mol. wt. acids 281.5, dissolved in 4.5 liters methanol (MeOH), cooled to −23°

Crystals, C_1, dissolved in 4.5 liters MeOH, cooled to −25° — Filtrate, F_1, 179 gm., I No. 102.3

C_2, redissolved in 4 liters MeOH, cooled to −25° — F_2, 16 gm., I No. 101.3

C_3, methyl stearate, 29 5 gm., I No. 0 4, m.p. 37.5° — F_3, 1.0 gm., I No. 63.7

$F_1 + F_2 + F_3$ (195 gm.), dissolved in 4 liters MeOH, cooled to −60°

C_5, redissolved in 4 liters MeOH, cooled to −60° — F_5, I No. 152 7, 30 gm.

C_6, 137 gm., I No. 83.6, redissolved in 4 liters MeOH, cooled to −60° — F_6, 18 gm., I No 147

C_7, redissolved in 4 liters MeOH, cooled to −60° — F_7, 5.5 gm., I No. 109.8

C_8, 125 gm. methyl "oleate," I No. 83.4 (theory 85.6), purity 97.4% — F_8, 3.5 gm., I No. 70.8 (I No. below 85 shows all linoleate removed)

Isolation of Oleic Acid from Linseed Oil

Several earlier reports of the composition of the fatty acids of linseed oil showed contents of oleic acid amounting to 6 to 18 per cent (20–23). Painter and Nesbitt (24), in analyzing the glycerides of this oil by the iodine-thiocyanogen equations, found somewhat higher values ranging from 12 to 38 per cent. There is no question that in some specimens of linseed oil, oleic acid is practically a minor component acid. So far as we know, the actual isolation of oleic acid from this oil has not been previously attempted.

CHART II

Crystallization of Oleic Acid from Lard

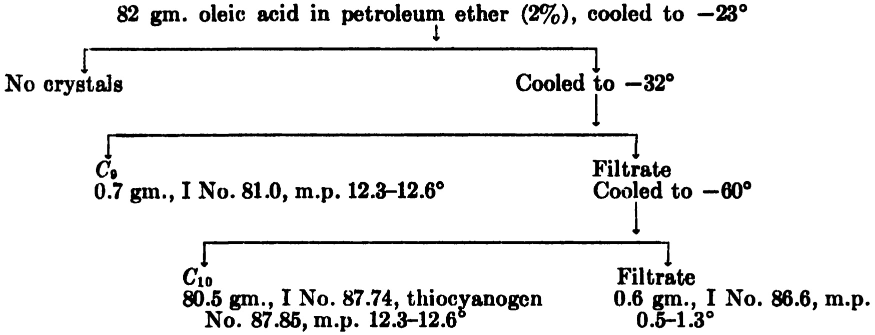
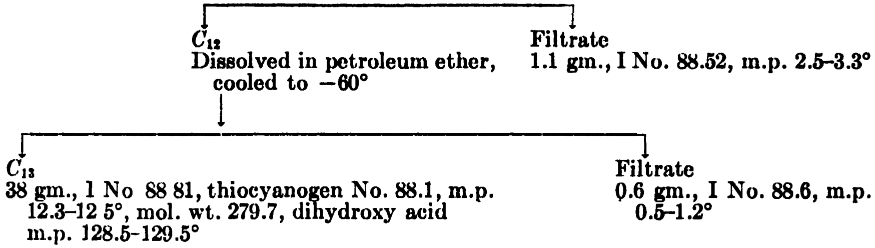

82 gm. oleic acid in petroleum ether (2%), cooled to −23°
No crystals
Cooled to −32°
C_9
0.7 gm., I No. 81.0, m.p. 12.3–12.6°
Filtrate
Cooled to −60°
C_{10}
80.5 gm., I No. 87.74, thiocyanogen No. 87.85, m.p. 12.3–12.6°
Filtrate
0.6 gm., I No. 86.6, m.p. 0.5–1.3°

CHART III

Recrystallization of C_{10}


46.5 gm. C_{10} dissolved in 1060 cc. ether, cooled to −40°
C_{11}
7 gm., I No. 87.2, thiocyanogen No. 87.7, m.p. 12.5–13.0°
Filtrate
Ether removed, dissolved in petroleum ether (about 4%), cooled to −60°
C_{12}
Dissolved in petroleum ether, cooled to −60°
Filtrate
1.1 gm., I No. 88.52, m.p. 2.5–3.3°
C_{13}
38 gm., I No. 88.81, thiocyanogen No. 88.1, m.p. 12.3–12.5°, mol. wt. 279.7, dihydroxy acid m.p. 128.5–129.5°
Filtrate
0.6 gm., I No. 88.6, m.p. 0.5–1.2°

The C_{18} esters of linseed oil consist of methyl stearate, oleate, linoleate, and linolenate, the last in preponderant amount. In the preparation of these esters there was some question concerning the possibility of distilling them without serious alteration. We are including, therefore, the distillation data in Table V.

The separation described in Table V was extremely efficient. Most of the previous analyses of linseed oil have not included values for other series than the C_{18}. Griffiths *et al.* (23), however, did report 5.4 per cent of palmitic, 0.2 per cent myristic, and 0.6 per cent arachidic acids. The molecular weights in Table V confirm the presence of these other carbon series. The residue, Fraction A-5, 9.2 gm., of molecular weight 285.2, is clear evidence that the distillation was carried out without appreciable polymerization, and further confirms the fact that only traces of C_{20} acids are found in this oil. The iodine number, 9.6, of the C_{16} fraction, A-2, is an indication of the probable presence of hexadecenoic acid in this oil.

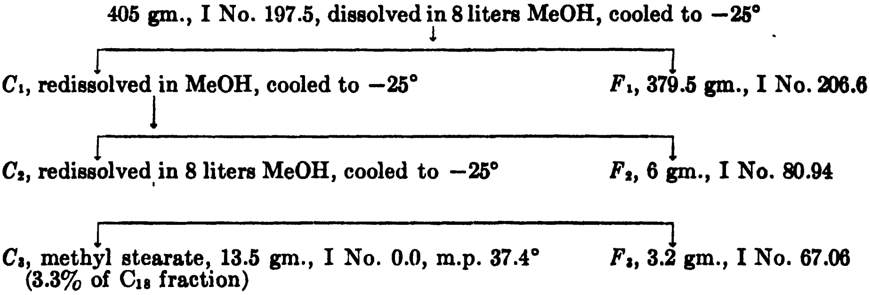
TABLE V

Analytical Constants of Various Fractions of Linseed Oil

Fraction	B p	Weight	Mol. wt.		I No.	Probable series
			Esters	Acids		
	°C	gm				
A-1	118 -120.5	5.7	253.3	239.3	1.33	C_{14}–C_{16}
A-2	120.5–125	37.9	270.1	256.1	9.56	C_{16}
A-3	125 -136	15.7	284.3	271.3	120.8	C_{16}–C_{18}
A-4	136 -140	695.6	294.5	280.5	197.5	C_{18}
A-5	Residue	9.2	299.2	285.2	110.6	C_{18}
Total		764.1				

CHART IV

Removal of Methyl Stearate from C_{18} Esters of Linseed Oil



405 gm., I No. 197.5, dissolved in 8 liters MeOH, cooled to −25°

C_1, redissolved in MeOH, cooled to −25°

F_1, 379.5 gm., I No. 206.6

C_2, redissolved in 8 liters MeOH, cooled to −25°

F_2, 6 gm., I No. 80.94

C_3, methyl stearate, 13.5 gm., I No. 0.0, m.p. 37.4° (3.3% of C_{18} fraction)

F_3, 3.2 gm., I No. 67.06

The isolation of methyl oleate from Fraction A-4 of Table V was carried out in three series of crystallizations as noted in Charts IV, V, and VI.

CHART V

Removal of Methyl Oleate from More Unsaturated Esters

200 gm. of F_1, I No. 206.6
in 4 liters MeOH, cooled to −20°

- C_4, 3.5 gm., I No. 68.8
- F_4, cooled to −39°
  - C_5, 1.0 gm., I No. 137.6
  - F_5, cooled to −48°
    - C_6, 31.5 gm., I No. 98.6
    - F_6, cooled to −55°
      - C_7, 10.5 gm., I No. 116.1
      - F_7, cooled to −60°
        - C_8, 2.5 gm., I No. 206.7
        - F_8, 147.5 gm., I No. 211.

Simplified Procedure

177 gm. F_1 fraction, I No. 206.6, dissolved in 2.5 liters MeOH, cooled to −20°

- C_4, 2.5 gm., I No. 71.7
- F_4, cooled to −55°
  - C_7, 48 gm., I No. 122.9
  - F_7, 126 gm., I No. 239.7

CHART VI

Purification of Methyl Oleate Fraction

Combined C_5 through C_7, 89.5 gm., I No. 120.5 (calculated), dissolved in 3.25 liters acetone, cooled to −70°

- C_1, redissolved in 3.25 liters acetone, cooled to −67°
  - C_2, 57 gm., I No. 81.8, dissolved in 3.25 liters acetone, cooled to −67°
    - C_3, 47.5 gm., I No. 80.7, dissolved in 2 liters MeOH, cooled to −32°
      - C_4, 1.2 gm., I No. 76.35
      - F_4, cooled to −63°
        - C_5, 36.5 gm., I No. 86.64
        - F_5, 1.0 gm., I No. 88.61
    - F_3, 3.8 gm., I No. 63.46
  - F_2, 5 gm., I No. 103.3
- F_1, 25.5 gm., I No. 200

The methyl oleate, C_5 of Chart VI, was converted into oleic acid which was recrystallized from petroleum ether as described in Chart VII.

The final product, C_2 of Chart VII, apparently was one of the best of our preparations of oleic acid as noted from the melting point and other constants. It was apparently identical with the oleic acid from olive oil.

CHART VII

Further Purification of Oleic Acid from Linseed Oil

Oleic acid, 19.0 gm., I No. 88.65, thiocyanogen No. 88.41, m.p. 13.05–13.30°, dissolved in petroleum ether (2%), cooled to −60°

C_1, redissolved in petroleum ether, cooled to −60°

F_1, 0.5 gm., I No. 89.6, m.p. 1.5–2.5°

C_2, 15.3 gm., I No. 90.06, thiocyanogen No. 89.13, m.p. 13.3–13.5°, mol. wt. 280.6

F_2, 0.4 gm., m.p. 4.75–5.5°

SUMMARY

1. Octadecenoic acids have been isolated by low temperature crystallization of the C_{18} methyl esters of a number of fats and oils and lipids of animal and vegetable origin. These octadecenoic acids have been compared with oleic acid made by similar methods from olive oil.

2. The octadecenoic acids of chicken fat, and of peanut, cottonseed, corn, and linseed oils appear to be identical with the oleic acid of olive oil. On the other hand, the octadecenoic acids of lard, beef tallow, beef adrenal phosphatides, pork liver lipids, human fat, and, to a somewhat lesser extent, soy bean and rape-seed oils appear to be mixtures of oleic acid with other isomeric octadecenoic acids, oleic acid being the principal component of these mixtures.

3. The evidence for these conclusions is based on the low melting points of the original preparations, the even lower melting points of certain filtrate fractions obtained by crystallization of these specimens, and the melting points of the dihydroxy acids.

4. So far as beef fat and lard are concerned, these results appear to confirm the previously reported presence of vaccenic acid in these fats. Our results and those previously reported point to the likelihood of a rather general occurrence of octadecenoic acids other than oleic in fats and other lipids of animal origin.

5. Details are presented of the isolation of oleic acid from lard and from linseed oil.

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PHOSPHORYLCHOLINE

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WITH A SECTION IN THE EXPERIMENTAL PART BY DOROTHY B. MUNDELL

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(Received for publication, April 17, 1944)

The synthesis of phosphorylcholine has been attempted already in a number of different ways. No less than five methods for its preparation have been developed. They include the phosphorylation of choline halide by (*a*) phosphorus oxychloride (1–3), (*b*) phosphorus pentoxide or phosphorus pentoxide and anhydrous phosphoric acid (2, 4–7), (*c*) ethyl metaphosphate (8), and the phosphorylation of ethylene chlorohydrin by (*d*) phosphorus oxychloride (2, 5, 9), (*e*) ethyl metaphosphate (8, 10, 11) with subsequent transformation of the phosphoryl ethylene chlorohydrin into phosphorylcholine by means of trimethylamine. The procedures give at best complex reaction mixtures from which the desired pure compounds can be isolated only with difficulty and in poor yields. An improved synthesis of phosphorylcholine was desirable which would supply the substance in a pure form for chemical and biochemical investigations.

Most complications encountered in the earlier procedures can be traced back to the use of unsuitable phosphorylating agents. Diphenylphosphoryl chloride, recently introduced for this purpose (12), was found eminently suited for the phosphorylation of choline and its use led to the development of a new procedure which has the distinct advantage of producing the ester in a pure state. The esterification (Equation A) is carried out in dry pyridine at room temperature and proceeds without the formation of secondary reaction products. Diphenylphosphorylcholine (I) is isolated from an aqueous solution of the reaction mixture in the form of its insoluble chloroaurate (approximately 90 per cent) from which it is recovered by treatment with finely divided metallic silver (13) in a yield of 98 per cent.

$$\text{(A)}\quad Cl(CH_3)_3N\cdot CH_2CH_2OH + Cl\text{—}PO(OC_6H_5)_2 + C_5H_5N \rightarrow$$
$$Cl(CH_3)_3N\cdot CH_2CH_2\text{—}O\text{—}PO(OC_6H_5)_2 + C_5H_5N\cdot HCl$$
$$\text{(I)}$$

$$\text{(B)}\quad Cl(CH_3)_3N\cdot CH_2CH_2\text{—}O\text{—}PO(OC_6H_5)_2 + 8H_2 \xrightarrow{Pt}$$
$$Cl(CH_3)_3N\cdot CH_2CH_2\text{—}O\text{—}PO(OH)_2 + 2C_6H_{12}$$
$$\text{(II)}$$

The phenyl groups of the phenyl ester are eliminated by catalytic hydrogenolysis in methanol with platinum and hydrogen at room temperature

(Equation B). Based on the amount of choline chloride used, the over-all yield of phosphorylcholine is usually about 88 per cent. When an aqueous solution of phosphorylcholine halide (II) is digested with barium carbonate, the barium salt is formed which, after removal of the excess barium carbonate, precipitates in glistening leaflets on the addition of ethanol. The substance is obtained immediately in analytically pure form. For analysis the substance was dried *in vacuo* (about 0.5 mm. of Hg) at 100° over phosphorus pentoxide to constant weight. The analytical values obtained for C, H, N, P, Cl, and Ba corresponded very well with those calculated for the anhydrous barium salt of phosphorylcholine.[1]

The investigation of phosphorylcholine by Plimmer and Burch (2) and Beznák and Chain (7) revealed the interesting fact that in contrast to most choline esters (*e.g.* lecithin, acetylcholine) the phosphoric acid ester is unusually resistant to acid and alkali.

Information pertaining to the chemical hydrolysis of phosphorylcholine being meager, further investigation was carried out with pure phosphorylcholine prepared as described below. For comparative purposes a similar study was made with phosphorylcholine prepared according to the directions of Plimmer and Burch.[2] The rates of hydrolysis of both phosphorylcholine preparations were investigated first at 100° ± 1° in 1 N hydrochloric acid (see Fig. 1, Curve 5). The results were roughly comparable with those obtained by Plimmer and Burch. The cleavage under these conditions was extremely slow, approximately 20 per cent in 12 hours [$k_{(\log 10)} = 1.3 \times 10^{-4}$]. The hydrolysis therefore was studied also in 2 N hydrochloric acid at 125° ± 1°. The adoption of these conditions, used previously with good results by Lohmann (14) and Meyerhof and Kiessling (15) in their investigations of the organic phosphates involved in carbohydrate metabolism, enabled us to carry out the complete hydrolysis of phosphorylcholine in a reasonable period of time and to compare its rate with those of other organic phosphates of similar constitution. The hydrolysis curves of our phosphorylcholine (Fig. 1, Curve 3) and of the preparation made by us according to Plimmer and Burch, but purified by preliminary partial hydrolysis, were found to follow closely the theoretical curve of a pseudounimolecular reaction $[k_{(\log\ 10)} = 1.16 \times 10^{-3}]$. Notwithstanding the conflicting analytical reports mentioned,[1] the pronounced similarity of both curves of hydrolysis confirms the identity of the phosphorylcholine preparations.

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[1] Plimmer and Burch (2) reported that their barium salt of phosphorylcholine which contained 4 molecules (16.88 per cent) of water when air-dried still retained water of crystallization in amounts varying between 8.4 and 4.55 per cent after having been dried *in vacuo* at 100° over phosphorus pentoxide. Such a retention of water of crystallization at 100° is at variance with our experience.

[2] The latter preparation was not obtained free from impurities. The presence of these became manifest when freshly prepared solutions of a repeatedly recrystallized barium salt of phosphorylcholine in 2 N hydrochloric acid developed inorganic phosphate on standing at room temperature. The lability of the impurity to acid suggested the presence of pyrophosphate, the formation of which may have been caused by the use of a mixture of phosphorus pentoxide and phosphoric acid. The contaminant, which if not removed would falsify the hydrolysis curve of phosphorylcholine, was destroyed by heating the solution of the barium salt in 2 N hydrochloric acid to 100° for a period of 3 hours. The resulting solution, free from easily hydrolyzable phosphate, was used for the investigation.

To earlier observers the insignificant hydrolysis of phosphorylcholine at 100° indicated an abnormally high acid stability but the newly determined rate of hydrolysis of phosphorylcholine at 125° placed the substance with regard to its acid stability in the same class as α-glycerophosphoric acid, β-glycerophosphoric acid, and glyceric acid-3-phosphoric acid (15). Since all four substances have similar stabilities but with the exception of phosphorylcholine are unable to form a betain, it is unlikely that this structure is responsible for the acid stability of phosphorylcholine as suggested by Beznák and Chain (7). The acid stability of phosphorylcholine probably is due to the fact that it is a mono ester of phosphoric acid.[3]

According to previous reports phosphorylcholine was neither saponified by 2 N alkali at 100° in 100 hours (2) nor by 10 N alkali at approximately 118–120° in 30 minutes (7). On raising the temperature of saponification in 2 N potassium hydroxide to 125° and extending the time to 50 hours, we found that 40 per cent of the organic phosphate was mineralized (Curve 2, Fig. 2). The saponification of phosphorylcholine at this temperature may, however, be accompanied by side reactions. The recognition of a strong odor of trimethylamine suggested that decomposition either of the ester or of free choline was occurring. However, under the conditions of the experiment it was impossible to determine the amount of trimethylamine formed or which of the two compounds gave rise to it. Some doubt may exist therefore as to whether the rate of saponification of phosphorylcholine is truly represented by Curve 2. However, the rate of saponification of α-glycerophosphate in 2 N potassium hydroxide at 125° ± 1°, although slightly higher than that of phosphorylcholine, was found to be of the same order of magnitude.

The action of true cholinesterase and pseudocholinesterase on phosphorylcholine has been investigated by Miss Dorothy B. Mundell. The results of this investigation, described in an appendix to the experimental part, indicate that the primary function of this compound is not as a derivative of choline but rather as a phosphoric acid ester. In support of this

[3] The great instability of both triose monophosphoric acids in dilute hot acids (16, 17), an apparent exception, in reality is due to decomposition into methylglyoxal and inorganic phosphate and not to hydrolysis.

view is the finding of Plimmer and Burch (2) that the compound is split by phosphatase.

The remarkable stability of phosphorylcholine to acid and alkali is of peculiar interest in view of the ease with which lecithin is broken down. The digestion of lecithin even with dilute acid or alkali at room temperature liberates choline (18). Phosphorylcholine, however, is hydrolyzed only when a strong acid or a strong alkali at a high temperature is employed. Thus it seems that the stability of an ester bond in an organic phosphate can vary considerably and that the sequence and the ease with which these linkages are opened depend on the degree of esterification and the nature of the substituents. In view of the importance of organic phosphates in metabolism the absolute and relative stabilities of secondary and tertiary esters deserve further investigation.

EXPERIMENTAL

Diphenylphosphorylcholine Chloroaurate—A mixture of 4.19 gm. (0.030 mole) of finely pulverized dry choline chloride, 8.65 gm. (0.033 mole) of diphenylphosphoryl chloride (12), and 26 gm. (0.33 mole) of dry pyridine[4] was shaken with several heavy glass rods in a closed bottle for 2 days at room temperature. The pasty mixture was transferred to a distilling flask and the excess pyridine was removed as thoroughly as possible by distillation under reduced pressure at 40°. In order to isolate the diphenylphosphorylcholine chloride the residue was dissolved in 650 cc. of water and a concentrated aqueous solution of 18.0 gm. (0.045 mole) of sodium chloroaurate ($2H_2O$) was added. The precipitate was filtered off with suction, washed with a small volume of cold water, and dried *in vacuo* over $CaCl_2$.

The mixture of the chloroaurates of pyridine and diphenylphosphorylcholine was extracted with boiling ethyl acetate. The filtrate was cooled and diphenylphosphorylcholine chloroaurate separated in bright yellow needles. This material usually melted incompletely at 108–118°, leaving a skeleton of a higher melting substance, presumably pyridine chloroaurate. Repeated recrystallization from ethyl acetate raised the melting point to 122–123°; yield 18.0 to 18.5 gm. (approximately 90 per cent). The product melting at this temperature gave a clear melt. Further recrystallization from methanol which yielded the chloroaurate in bright yellow platelets did not raise the melting point. The substance is soluble in cold dioxane and hot absolute methanol, slightly soluble in cold 99 per cent ethanol and boiling water. It is insoluble in hot benzene and boiling ether.

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[4] In order to obtain high yields of diphenylphosphorylcholine chloride a large excess of pyridine is necessary, since choline chloride is only slightly soluble in this solvent.

For analysis the diphenylphosphorylcholine chloroaurate was kept over phosphorus pentoxide *in vacuo* at room temperature for 12 hours.

$C_{17}H_{23}O_4NClP \cdot AuCl_3$ (675.5)
Calculated. C 30.25, H 3.43, N 2.08, Cl 21.00, P 4.59, Au 29.2
Found. " 30.20, " 3.10, " 2.02, " 20.80, " 4.50, " 29.7

Diphenylphosphorylcholine Chloride—The pure chloroaurate was decomposed with metallic silver according to the procedure of Dudley (13). A solution of 18.0 gm. of diphenylphosphorylcholine chloroaurate in 120 cc. of hot methanol was stirred with 60 gm. of finely divided silver until the solution became colorless (approximately 2 to 3 minutes). The inorganic material was filtered off, washed well with hot methanol, and the combined filtrate and washings were taken to dryness under reduced pressure. Yield 9.70 gm.; 98 per cent of theory. The hygroscopic ester is very soluble in water and alcohol but only slightly soluble in anhydrous acetone.

For analysis the substance was crystallized from anhydrous acetone containing 2.5 per cent methanol and dried *in vacuo* over phosphorus pentoxide at room temperature for 3 days. White needles, m.p. 133–134°.[5]

$C_{17}H_{23}O_4NClP$ (371.8)
Calculated. C 54.91, H 6.23, N 3.77, Cl 9.5, P 8.33
Found. " 54.70, " 6.31, " 3.57, " 9.3, " 8.13

Barium Salt of Phosphorylcholine Chloride—A solution of 9.70 gm. (0.026 mole) of diphenylphosphorylcholine chloride in 225 cc. of anhydrous methanol and 2.0 gm. of platinum oxide (Adams' catalyst) was shaken vigorously in an atmosphere of pure hydrogen at a pressure of approximately 40 to 50 cm. of water. The reaction mixture was kept cool (15–20°) by dropping ether on the reaction vessel. Absorption of the gas ceased when 4890 cc.[6] of hydrogen (normal temperature and pressure), *i.e.* the theoretical amount, were taken up. The catalyst was filtered off, washed with methanol, and the combined filtrate and washings were evaporated to dryness *in vacuo*. Yield 5.8 gm. (100 per cent) of phosphorylcholine chloride. The over-all yield, including this step, is about 88 per cent. The very viscous residue was dissolved in 65 cc. of water and was digested with an excess of barium carbonate on a hot water bath. The filtrate and washings from the undissolved barium carbonate were combined and concentrated to a volume of 30 cc. On dropwise addition of

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[5] This melting point is given with reservation, since the hygroscopicity of the substance makes it difficult to take without special precautions. The substance was dried in the melting point tube over phosphorus pentoxide *in vacuo* at room temperature for 3 days and the tube was sealed immediately on removal from the desiccator.

[6] Included is the amount of hydrogen required for the reduction of the catalyst.

180 cc. of 99 per cent ethanol the barium salt of phosphorylcholine separated in leaflets. The salt is readily soluble in water but insoluble in ethanol.

For analysis the salt was dried *in vacuo* (0.5 mm.) over phosphorus pentoxide at 100° for 16 hours.

$C_5H_{13}O_4NClPBa \cdot H_2O$ (373)
Calculated. C 16.10, H 4.05, N 3.75, Cl 9.51, P 8.31, Ba 36.83
$C_5H_{13}O_4NClPBa$ (355)
Calculated. C 16.90, H 3.69, N 3.94, Cl 9.99, P 8.74, Ba 38.67
Found. " 16.90, " 3.75, " 3.92, " 10.0, " 8.90, " 38.70

Acid Hydrolysis of Phosphorylcholine—A series of sealed off Pyrex test-tubes,[7] each containing 6 to 7 cc. of 0.00365 M solution of phosphorylcholine in 2 N HCl, was kept in a bath at 125° ± 1°. At intervals of 1 hour during the early stages of the hydrolysis and of 2 hours towards its end tubes were removed and their inorganic phosphorus was determined colorimetrically. King's procedure (19) in a slightly modified form was used. The addition of perchloric acid was omitted and the standards and samples were made up to contain equal amounts of hydrochloric acid (5 cc. of 2 N HCl per 15 cc.). The determination of the total phosphorus was carried out according to King's original procedure. The hydrolysis at 100° was carried out in essentially the same manner with a 0.03 M solution of phosphorylcholine in 1 N hydrochloric acid. The rates of hydrolysis of phosphorylcholine are shown in Fig. 1 (Curves 3 and 5).

Alkali Hydrolysis of Phosphorylcholine—The potassium salt of phosphorylcholine was prepared for the study of the hydrolysis of phosphorylcholine in potassium hydroxide solution.[8] A boiling solution of 50 mg. of potassium sulfate in 10 cc. of water was added to a hot solution of 90 mg. of barium phosphorylcholine in 10 cc. of water. The barium sulfate was filtered off and the aqueous solution was evaporated *in vacuo* to dryness. The residue was dissolved in 75 cc. of 2 N potassium hydroxide and a portion of this solution (55 cc.) was placed in a silver-lined copper pressure vessel.[9] The vessel was sealed and after being heated in a boiling water bath for 5 minutes it was transferred to an oven kept at 125° ± 1°. Aliquots (5 cc.) for the determination of inorganic phosphate were taken at the end of 4, 20, 32, and 47 hours.

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[7] The surface of the test-tubes was freed from material which develops a blue color with the phosphate reagent by treating the tubes with boiling 5 N hydrochloric acid for a period of 5 hours.

[8] Potassium hydroxide was chosen, because on the addition of perchloric acid most of the salt which interferes with color development is removed.

[9] An attempt to carry out the alkaline hydrolysis in sealed Pyrex test-tubes failed, because the amount of chromogenic material leached from the glass was variable and greatly exceeded the quantity derived by hydrolysis of organic phosphate.

To each of the aliquots 2.12 cc. of 60 per cent perchloric acid were added (0.92 cc. was required for neutralization) and the potassium perchlorate was filtered off. The precipitate was washed with three 1 cc. portions of water. The filtrate and washings of each aliquot were transferred quantitatively to a 15 cc. volumetric flask.

The total phosphorus in the alkaline solution of the potassium salt was determined by digesting 1.00 cc. of the mixture with 1.8 cc. of 60 per cent perchloric acid in the manner described by King. After the contents of the digestion tube were diluted with 3 cc. of water, the potassium perchlorate was filtered off and washed three times with 1 cc. of water. The filtrate and washings were collected in a 15 cc. volumetric flask.

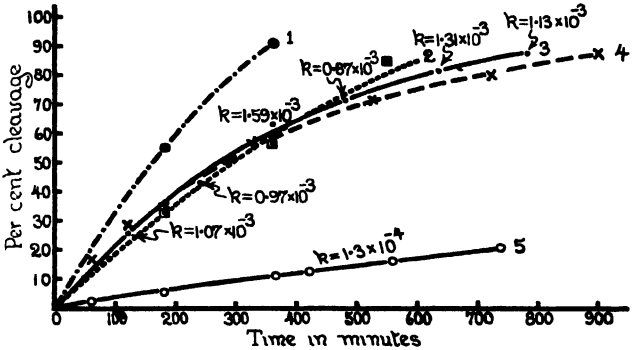

FIG. 1. Hydrolysis curves of (Curve 1) glyceric acid-3-phosphoric acid in 2.035 N HCl at 124°, (Curve 2) β-glycerophosphoric acid in 2.035 N HCl at 124°, (Curve 3) phosphorylcholine in 2.0 N HCl at 125° ± 1°, (Curve 4) α-glycerophosphoric acid in 2.14 N HCl at 127°, (Curve 5) phosphorylcholine in 1.0 N HCl at 100°. Curves 1, 2, 4 were constructed from the data given by Meyerhof and Kiessling (15). The hydrolysis curve of phosphorylcholine prepared by us according to the procedure of Plimmer and Burch is identical with Curve 3. The values of *k* were calculated with the minute as the unit of time.

To make the conditions in the standards comparable with those in the samples a special dilute phosphate standard had to be prepared. 2.50 cc. of the stock solution of KH_2PO_4 (2.1935 gm. per 500 cc.) were diluted to 250 cc. with 2 N KOH. To 5 cc. of this solution 2.12 cc. of 60 per cent perchloric acid were added. The potassium perchlorate was filtered off, washed with three 1 cc. portions of water, and the filtrate and washings were collected in a 15 cc. volumetric flask.

Colors were developed in the standard and test solutions by adding 1 cc. of 5 per cent ammonium molybdate and 0.5 cc. of aminonaphtholsulfonic acid reagent. The volumes were made up to 15 cc. with water. After

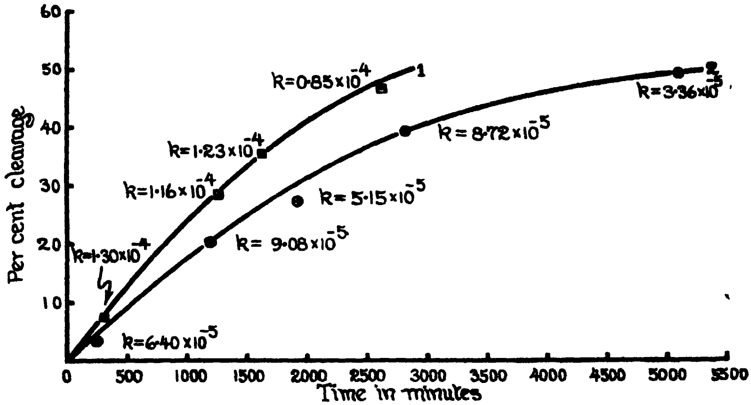

FIG. 2. Hydrolysis curves of (Curve 1) α-glycerophosphoric acid in 2 N potassium hydroxide at 125° ± 1°, (Curve 2) phosphorylcholine in 2 N potassium hydroxide at 125° ± 1°.

TABLE I

Hydrolysis of Phosphorylcholine by Cholinesterase

Type and quantity of purified cholinesterase acting on 275 mg. per cent solution of phosphorylcholine		Time	Amount of inorganic P* found in experimental solutions		Theoretical amount of phosphorus in experimental solutions
			Without eserine	With 5 × 10^{-5} M eserine	
		hrs.	*mg.*	*mg.*	*mg.*
Pseudocholinesterase	From dog pancreas (21),† 1.4 units‡ $Q_{\text{Ach.}}$ 30,000	0	0		
		2	0.105		2.25
		6	0.363	0.356	
	From horse serum (22),† 74 units $Q_{\text{Ach.}}$ 54,000	0	0	0	
		6	0	0	1.12
True cholinesterase	From human red blood cells (20),† 18.2 units $Q_{\text{Ach.}}$ 500	6	0.01 (*ca.*)	0.01 (*ca.*)	1.0
	From human red blood cells 61 units $Q_{\text{Ach.}}$ 500	7½	0.03 "	0.03 "	1.0

* Determined according to King (19).

† Bibliographic reference number.

‡ 1 unit of cholinesterase is defined as the amount of enzyme capable of hydrolyzing 7.3 mg. of acetylcholine at a substrate concentration of 1 per cent acetylcholine for pseudocholinesterase and 10 mg. per cent of acetylcholine for true cholinesterase.

15 minutes, the unknowns were compared colorimetrically with the standard. The hydrolysis curve is given in Fig. 2, Curve 2.

The rate of hydrolysis of α-glycerophosphoric acid in 2 N potassium hydroxide at 125° ± 1° was determined in the same manner (Fig. 2, Curve 1).

Cholinesterase and Phosphorylcholine

BY DOROTHY B. MUNDELL

Purified preparations of true and pseudocholinesterase (20) were allowed to react with phosphorylcholine, the experiments being carried out at 37.5° and pH 7.4 (0.025 M bicarbonate in equilibrium with 5 per cent CO_2 in N_2).

From Table I it is seen that the small amounts of P found were the same whether or not eserine was present. Since cholinesterase is inhibited by eserine, any action on phosphorylcholine was probably due to some phosphatase in the purified preparations.

Conclusions—Neither true nor pseudocholinesterase is able to hydrolyze phosphorylcholine. This confirms the results of Bezmák and Chain (7) who found no action of serum cholinesterase on phosphorylcholine by their method.

SUMMARY

1. A new synthesis of phosphorylcholine is described in which no secondary reaction products are formed. The intermediates and the end-product are obtained readily in a pure condition and in excellent yields.

2. The synthesis involves three steps: (*a*) phosphorylation of choline halide with diphenylphosphoryl chloride in pyridine and isolation of diphenylphosphorylcholine as chloroaurate; (*b*) decomposition of the chloroaurate with metallic silver to obtain the diphenylphosphorylcholine; (*c*) catalytic hydrogenolysis of the phenyl ester to phosphorylcholine and the preparation of the barium salt.

3. The rates of hydrolysis of phosphorylcholine in acid at 100° and 125° and alkali at 125° were determined and found comparable with those of α-glycerophosphoric acid, β-glycerophosphoric acid, and glyceric acid-3-phosphoric acid.

4. True cholinesterase and pseudocholinesterase do not hydrolyze phosphorylcholine.

Grateful acknowledgment is made to the Banting Research Foundation for grants to two of the authors (C. S. McA. and D. B. M.). The micro combustions were carried out by Mrs. E. Mason, to whom the authors express their thanks.

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TEMPERATURE ACTIVATION AND INACTIVATION OF THE CRYSTALLINE CATALASE-HYDROGEN PEROXIDE SYSTEM

BY IRWIN W. SIZER

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(Received for publication, April 22, 1944)

The many studies on the action of temperature changes upon the catalase-H_2O_2 system have been reviewed by Zeile (25). Some workers have found that this reaction, like many other enzyme-catalyzed reactions, increases with temperature in accordance with the Arrhenius equation (*cf.* Sizer (16)) with corresponding activation energies varying from 2600 to 6200 calories per gm. molecule. Others (*cf.* Williams (24)) have found that the activation energy changes both with temperature and pH, while Morgulis *et al.* (12) came to the conclusion that the optimum temperature for catalase action is at 2° instead of 40–50° as reported by others. Above the optimum temperature the rate of catalase inactivation increases rapidly with temperature, and the corresponding activation energies are between 30,000 and 50,000 calories per gm. molecule. Part of the difficulty in procuring significant data on the effects of temperature on the catalase-H_2O_2 system arises from the fact that, due to its strong oxidizing power, H_2O_2 is a very toxic substance and rapidly inactivates the catalase. To overcome this major difficulty in the present investigation very dilute peroxide solutions at optimum pH have been employed, and manometric data on oxygen evolution from peroxide during only the initial few minutes of the reaction have been used, during which time the destruction of the enzyme by the dilute peroxide solution is almost negligible. Another difficulty has been that all previous workers have utilized impure enzyme preparations from a variety of sources; it seems possible that such impurities have exerted some influence on the kinetics of the reaction. In this study use has been made of once recrystallized beef liver catalase. The inactivation of catalase by heat has been investigated both in the presence and absence of substrate.

Methods

The catalase used in these experiments was prepared from beef liver and was once recrystallized according to the method of Sumner and Dounce (21). A saturated solution of crystalline catalase was kept in the refrigerator and for each series of experiments a suitable dilution of the enzyme was made. Although several different preparations of crystalline catalase were made from liver, no important difference in their behavior was noticed. The reaction was followed by measuring oxygen evolution from H_2O_2 with a Warburg-Barcroft manometer, with a reaction vessel having a single side arm. In the cup were placed 1 ml. of suitably diluted catalase, 1 ml. of 0.03 M phosphate buffer, pH 6.9, and in the side arm, 1 ml. of 0.03 M H_2O_2. The compensating vessel contained the same, but with water substituted for enzyme. After 2 minutes of shaking (110 strokes per minute) in the water bath (temperature control = $\pm 0.02°$) the stop-cocks were closed and the reaction initiated by tipping the peroxide into the buffered catalase solution. Usually about ten readings were made on the manometers at each temperature at intervals determined by the speed of the reaction.

A fresh H_2O_2 solution was prepared for each series of temperature studies run on a given day. The peroxide solutions were checked by titration with permanganate and it was found that the peroxide concentration did not change during the 8 hours of the experiment. Preliminary studies were performed in which the permanganate method of titrating the residual peroxide was used to indicate the course of the reaction. This method proved much less sensitive than the manometric method and required higher concentrations of catalase and peroxide, and was therefore not used.

Preliminary experiments indicated that the rate of destruction of H_2O_2 increases with temperature up to about 53°, above which the rate decreases rapidly owing to the destruction of the catalase by heat. For experimental purposes the temperature range was divided into two portions which were studied separately: (*a*) the range of 0–40° in which the enzyme-catalyzed reaction predominates, and (*b*) the range from 57–68° in which the destruction of the enzyme is the major reaction. A given range of temperature was studied on a single day with the same enzyme and peroxide solutions throughout. Two different techniques were employed in the investigation of the heat inactivation of catalase. The first technique is the same as that used at lower temperatures and involves adapting to temperature for 2 minutes before the peroxide is added. In this method (essentially that used by Johnson *et al.* (7) for luciferase) the observed temperature effects represent a combination of the acceleration of the enzyme-catalyzed reaction with rise in temperature and the retardation of the decomposition of H_2O_2 with rise in temperature due to increased enzyme destruction caused by the higher temperature. In the second technique 1 ml. of buffer after adjustment to temperature is added to 1 ml. of enzyme solution and incubated at the desired temperature for 5 minutes. The solution is then cooled rapidly on ice, and the residual catalase activity determined at 35° by adding 1 ml. of 0.03 M H_2O_2 and following the reaction in the usual way. Data on volumes of oxygen evolved at different temperatures were converted to standard temperature before being analyzed.

Results

The temperature range from 2–40° was investigated at 5° intervals in five different series of experiments. At each temperature, including those at which inactivation of catalase occurs, the reaction follows zero order kinetics for a brief period. Typical data are shown in Fig. 1, where oxygen evolution is plotted against elapsed time in minutes. After 200 to 300 c.mm. of gas have been evolved, the plotted points fall off from the curve, suggesting some destruction of the enzyme by the peroxide. Most workers (*cf.* Sumner (20)) have considered this reaction monomolecular, but in the data presented in Fig. 1 the reaction is of zero order rather than monomolecular. The difference is probably related to the fact that in the present study different techniques and concentrations of reactants have been employed and attention is focused on the very early part of the reaction, before any appreciable catalase inactivation has occurred.

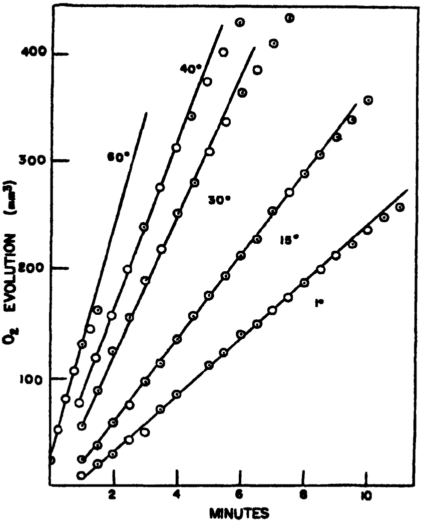

Fig. 1. Oxygen evolution from an 0 01 M H_2O_2 solution at pH 6.9 is plotted against elapsed time in minutes. The reaction catalyzed by a dilute solution of crystalline catalase is linear for a brief period at each temperature.

The rate at each temperature was calculated from the slope of the straight line which best fitted the plotted points. When log rate is plotted against the reciprocal of the absolute temperature (Fig. 2), the data are best fitted by a straight line in the temperature range from 2–40°. The slope of the line corresponds to an activation energy[1] of 4200 calories per gm. molecule. Similar results were obtained in the other series of experiments. At temperatures at which neither the enzyme-catalyzed reaction nor the destruction of the enzyme predominates, it was experimentally difficult to obtain quantitative data. Above 55° the latter phenomenon predominates and the reaction again can be followed quantitatively. From Fig. 2 it is apparent that the rate of peroxide decomposition by catalase decreases sharply with rise in temperature above 55° owing to the high temperature coefficient for inactivation of the enzyme. The heat inactivation of catalase increases with temperature in accordance with the Arrhenius equation; the slope of the line in the graph corresponds to an apparent activation energy of 51,000 calories. Since this value is the resultant of the effects of temperature upon both the H_2O_2-catalase system and the inactivation of the enzyme, the true value for the latter is 51,000 + 4200 or 55,200 calories.

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[1] μ in the Arrhenius equation, $\mu = 4.6 \dfrac{(\log k_2 - \log k_1)}{(1/T_1 - 1/T_2)}$ where k_1 and k_2 are rates at the absolute temperatures T_1 and T_2.

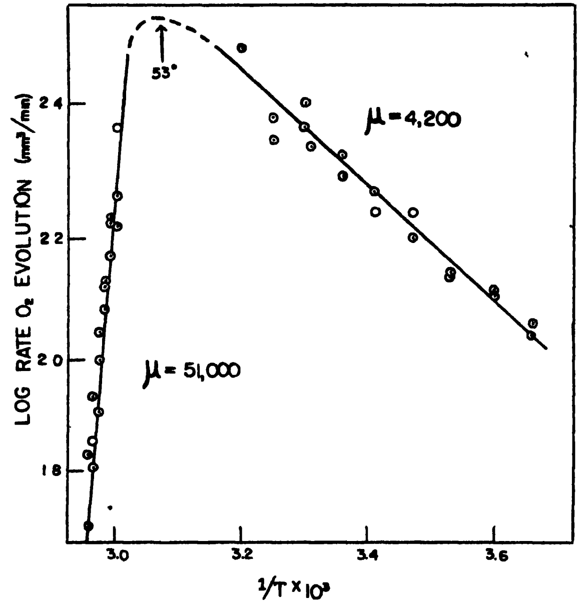

FIG. 2. Log rate of decomposition of H_2O_2 (as measured by oxygen evolution) catalyzed by crystalline catalase is plotted against $1/T$. The rate increases in accordance with the Arrhenius equation (μ = 4200 calories) up to about 53°, above which it decreases owing to heat inactivation of catalase, in accordance with the Arrhenius equation (μ = 51,000 calories uncorrected).

In the second method of studying the inactivation of catalase by heat, the enzyme and buffer were incubated at a given temperature for 5 minutes and the residual activity determined manometrically after addition of

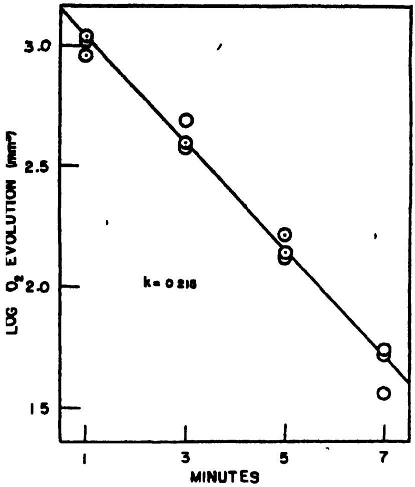

FIG. 3. The log rate of inactivation of catalase at pH 6.9 by heat at 62° is plotted against elapsed time. The inactivation is monomolecular. k = 0.215 ($\log_{10}$, minutes).

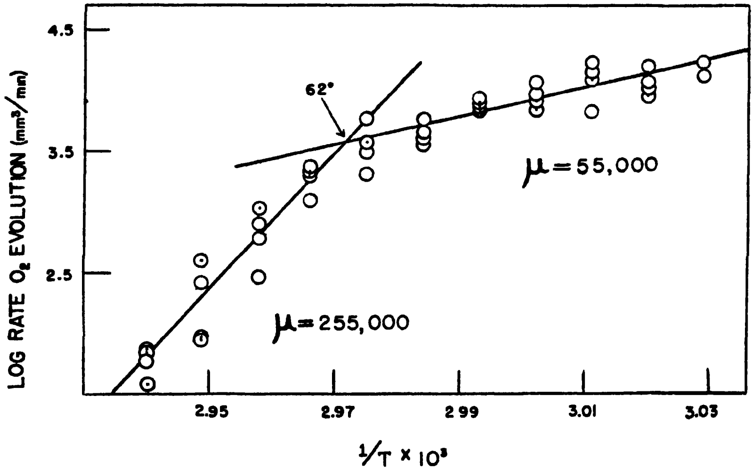

FIG. 4. Heat inactivation of catalase. After 5 minutes incubation at pH 6.9 at each temperature the residual catalase activity is measured at 35° by determining the rate of liberation of O_2 from H_2O_2. The log rate is plotted against $1/T$. Below 62° the activation energy for the heat destruction of catalase is 55,000 calories; above this temperature it is about 255,000 calories.

H_2O_2 at 35°. In studying the kinetics of inactivation of catalase we found that at a given temperature inactivation increases with incubation time, in accordance with the unimolecular equation (Fig. 3). The inactivation reaction as a function of temperature follows the Arrhenius equation up to about 62° (Fig. 4) and is characterized by an activation energy of about 55,000 calories, which is the same as the corrected value obtained by the first technique. It should be pointed out, however, that H_2O_2, a strong oxidizing agent, is present in the first technique but not in the other, but despite this the same activation energy for heat inactivation is obtained by both methods in the temperature range below 62°. Above 62° (studied only by the second method), however, the temperature effect abruptly changes (Fig. 4) and from 62–68° the activation energy becomes about 255,000 calories. This value as well as the critical temperature varies considerably from one experiment to the next and, owing to experimental difficulties, cannot be measured with any great certainty.

In some experiments, but not in all, the activity was somewhat higher after 5 minutes incubation of enzyme and buffer at 57° than for the unheated control, when the activity of both was measured at 35°. This "temperature activation" of the enzyme is probably due to the destruction of traces of catalase inhibitor which may have been present. These results are consistent with reports that a catalase inhibitor, unstable at 40–60°, accompanies catalase in blood, fat, and liver (1, 19). Neither this temperature activation at 57° nor the temperature inactivation of catalase at higher temperatures appears to be reversible.

The entropy change which characterizes the catalytic breakdown of H_2O_2 by catalase can be calculated from the equation of Glasstone, Laidler, and Eyring (4)

$$k = k^1 \frac{KT}{h} e^{-E/RT} e^{\Delta S/R}$$

where k is the velocity constant, k^1 is the transmission coefficient which can usually be assumed to be unity, K is Boltzmann's constant, h is Planck's constant, E is the activation energy which in enzyme reactions is essentially equal to μ, ΔS is the entropy of activation, and the other symbols have their usual meaning. At 0° this equation reduces to

$$k_0 = 5.7 \times 10^{13} e^{-E/RT} e^{\Delta S/R}$$

where k_0, the velocity constant at 0°, is calculated to the base e, and is expressed in seconds per mole of enzyme per liter. The k_0 of crystalline beef liver catalase can be determined from the "*Kat. f.*"[2] measured at 0° under standard conditions. Some uncertainty exists concerning the activity of crystalline beef liver catalase, but since Sumner and his associates (23) were unable to demonstrate a *Kat. f.* greater than 40,000, it seems permissible to accept this figure. To calculate k_0 from *Kat. f.* it is necessary to change from $\log_{10}$ to $\log_e$, from minutes to seconds, and from gm. of catalase per ml. to moles of catalase per liter.

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[2] "*Kat. f.*" = K/(gm. of enzyme per ml.), where K is the monomolecular velocity constant for the breakdown of H_2O_2 calculated with $\log_{10}$ and minutes.

$$k_0 = \frac{40{,}000 \times 2.3}{60 \times \dfrac{1000}{225{,}000}} = 3.4 \times 10^5 \text{ liter mole}^{-1} \text{ sec.}^{-1}$$

where 225,000 is the molecular weight of catalase (20). From the above it is clear that 1 molecule of catalase will catalyze the breakdown of 3.4×10^5 molecules of H_2O_2 per second. This figure is to be compared with the estimate of k_0 of Stern (18) of 10^5, and of Haldane[3] (6) of 5.4×10^4 liter mole^{-1} sec.$^{-1}$. Substitution of the calculated value of k_0 in the equation leads to a value of ΔS of

$$3.4 \times 10^5 = 5.7 \times 10^{13} \times e^{-4200/2 \times 273.1} \times e^{\Delta S/2}$$

$$\Delta S = -23 \text{ calories per degree per mole}$$

The negative sign indicates a decrease in entropy or a decrease in randomness during the catalysis; this might be conceived of as related to the high degree of orientation between the catalase and H_2O_2, which may be required in the formation of the enzyme-substrate compound.

TABLE I

Velocity Constant, Activation Energy, and Entropy of Activation for Enzyme-Catalyzed Reaction and for Heat Inactivation of Enzyme

Reaction	k_0	μ	ΔS
	l mole^{-1} sec.$^{-1}$	*calories*	*calories*
Action of catalase on peroxide	3.4×10^5	4,200	-23
Inactivation of catalase below 62°	6.8×10^{-11}	55,000	$+90$
" " " above 62°	2.4×10^{-39}	255,000	$+690$

In a similar manner the entropy of activation for the heat inactivation of catalase may be calculated for the two different types of inactivation encountered above and below 62° (*cf.* Fig. 4). For both types the monomolecular velocity constant at 62° is 0.215 (*cf.* Fig. 3), which, when converted to $\log_e$ and seconds, becomes 8.25×10^{-3}. If this value is substituted in the Arrhenius equation, it is found that $k_0 = 6.8 \times 10^{-11}$ for heat inactivation below 62° and 2.4×10^{-39} for heat inactivation above 62°. If these values are used in the equation for ΔS, it is found that the corresponding values of ΔS are $+90$ and $+690$ calories per degree per mole (see Table I). Such high values for the entropy of activation associated with heat inactivation of enzymes commonly characterize the heat denaturation of proteins (16) and suggest a similar mechanism for the action of heat in the two phenomena.

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[3] The latter figure was calculated from Zeile's data and was based on the assumption that all the Fe present in the crude enzyme preparation was in the catalase molecule, and that each catalase molecule contains 1 Fe atom instead of 4.

From the data available from this study of temperature activation of the catalase-H_2O_2 system, it is possible to calculate the rate of the reaction, assuming that H_2O_2 decomposition results from activated collisions between catalase and H_2O_2 molecules, by use of the methods of Haldane (6) and Moelwyn-Hughes (9–11). This calculated rate may then be compared with the experimental rate if for the calculated rate the modified Arrhenius equation is used,

$$k = Ze^{-\mu/RT}$$

where Z is the number of collisions and $e^{-\mu/RT}$ is the probability that a particular collision will have the necessary energy for reaction to occur.

In the first method of calculating the collision frequency, Z, the large catalase molecule is assumed to be a wall against which the H_2O_2 molecules collide. Use is made of the expression

$$Z = \frac{3}{13} \bar{u} n_1 n_2 \pi \sigma^2$$

where Z is the number of collisions per second per ml., $\bar{u}$ is the velocity (cm. per second) of the H_2O_2 molecule, $n_1 n_2$ the numbers of H_2O_2 and catalase molecules, respectively, per ml., and σ is the diameter of the catalase molecule.

$$\bar{u} = \sqrt{\frac{3RT}{M_{H_2O_2}}} = \sqrt{\frac{3 \times 8.315 \times 10^7 \times 273.1}{34.01}} = 4.4 \times 10^4 \text{ cm. per sec.}$$

$n_1 = 10^{-5} \times 6.02 \times 10^{23} = 6 \times 10^{18}$ per ml.

$n_2 = 1.72 \times 10^{12}$ per ml. (calculated from *Kat. f* = 40,000)

$\sigma = \sqrt[3]{\frac{6v}{\pi}} = 8.03 \times 10^{-7}$ cm.,[4] v, the volume of 1 catalase molecule = 225,000/dN, where 225,000 is the molecular weight, N is Avogadro's number, and d, the density of catalase, is 1.37 (22)

Substituting these values in the foregoing equation leads to a value of Z of 2.1×10^{23} collisions per ml. per second. Since only $e^{-\mu/RT}$ of these are activated collisions, the number of activated collisions per ml. per second $= 2.1 \times 10^{23} \times 4.7 \times 10^{-4} = 9.9 \times 10^{19}$.

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[4] This calculation of σ assumes the catalase molecule to be spherical, but its shape is not known. One may also calculate σ from the empirical equation of Moelwyn-Hughes, which has been found to be generally applicable. $\sigma = 1.33 \times 10^{-8} \times V_m^{\frac{1}{3}}$ where V_m is the molecular volume in ml. per mole. Use of this equation leads to a value of 8.1×10^{-7} cm. for σ of catalase, which is the same as that obtained by the method used above.

The experimental value for the number of peroxide molecules destroyed per ml. per second is obtained by multiplying the experimental monomolecular velocity constant (which gives the fraction destroyed each second) by the number of peroxide molecules per ml.

$$
\begin{aligned}
k_{\log_e} \text{ sec.}^{-1} &= 0.03838 \times k_{\log_{10}} \text{ min.}^{-1} \\
&= 0.03838 \times 0.0316 \ (cf.(21)) \\
&= 1.21 \times 10^{-3}
\end{aligned}
$$

$$
\begin{aligned}
H_2O_2 \text{ molecules destroyed per ml. per sec.} &= 1.21 \times 10^{-3} \times 10^{-5} \times 6.02 \times 10^{23} \\
&= 7.3 \times 10^{15}
\end{aligned}
$$

$$\frac{\text{Activated collisions}}{\text{Molecules destroyed}} = \frac{9.9 \times 10^{19}}{7.3 \times 10^{15}} = 1.4 \times 10^{4}$$

From this calculation it appears that only 1 in 14,000 activated collisions between catalase and H_2O_2 actually results in the decomposition of the peroxide.

Another method of calculating the rate is to make use of the gas collision formula in which Z, the total number of collisions between enzyme and substrate per ml. per second, is

$$Z = n_1 n_2 \left(\frac{\sigma_1 + \sigma_2}{2}\right)^2 \left(8\pi RT\left(\frac{1}{M_1} + \frac{1}{M_2}\right)\right)^{\frac{1}{2}}$$

where n^1 = number of molecules of H_2O_2 per ml. = 6.02×10^{18}*
n_2 = " " catalase molecules " " = 1.72×10^{12}*
σ_1 = diameter of peroxide molecule = 6×10^{-8} cm. (15)
σ_2 = " " catalase " = 8×10^{-7} " *
R = 8.315×10^7 ergs per degree
T = 273.1°, absolute
M_1 = molecular weight of peroxide = 34.02 gm.
M_2 = " " " catalase = 225,000 gm.

$Z = 2.5 \times 10^{23}$ collisions per ml. per second. The fraction of these with energy greater than 4200 calories is 4.7×10^{-4}; therefore the number of activated collisions = 1.16×10^{20} per ml. per second.

The experimental rate of the reaction expressed in the same units is 7.3×10^{15} per ml. per second (see above) or 1.6×10^{4} times slower than the theoretical rate. A comparison of the values of the experimental rate obtained by considering the catalase a stationary wall against which the small substrate molecules collide and by the method of considering random collisions between moving enzyme and substrate molecules indicates that essentially the same values are obtained for the theoretical rate by both methods.

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* See the calculations above.

DISCUSSION

The activation energy of 4200 calories for the enzyme-catalyzed reaction agrees well with the value obtained by Nosaka (13) and is of the same order of magnitude as the several values reported by Williams (24) for different temperatures. Except for tyrosinase (5) this value is the smallest activation energy reported (*cf.* (16)) and there is the possibility that diffusion (characterized by a low activation energy) might be the limiting factor in the catalase-peroxide reaction. Moelwyn-Hughes (10), however, considers this possibility unlikely in the light of other properties of the catalase system.

The high value of 55,000 calories for heat inactivation of catalase is similar to the values reported previously (12, 14) and, together with the value of 255,000 calories, is in the range of values which characterize heat inactivation of enzymes and heat denaturation of proteins (*cf.* 16)). The high positive values of ΔS of +90 and +690 associated with the heat inactivation of catalase are similar to those obtained for other enzymes and proteins (*cf.* (16)) and lend support to the concept that heat inactivation of catalase corresponds to the heat denaturation of the catalase molecule. These high values suggest that in the activated complex new degrees of freedom become active and present the possibility that enzyme inactivation (denaturation) is essentially a dissociation process.

The value of −23 calories per degree per mole for the entropy of activation of the enzyme-catalyzed reaction is interpreted as indicating a loss in randomness when the catalase combines with peroxide to form an intermediary compound and accounts for the fact that the measured rate is slower than one would expect from merely considering the activation energy. This value is much lower than the average value of −59 calories for ΔS calculated by Stearn (17) from data in the older literature, and is similar to the values recently reported by Butler (2) for certain crystalline proteases. As Butler points out, these small negative values for ΔS are reasonably close to the range usually taken as representing normal chemical reactions.

If one, instead of using the viewpoint of statistical mechanics, adopts the collision hypothesis, it is clear from the calculations that the experimental rate is 1.6×10^4 times slower than the theoretical rate. In these calculations it is assumed that every activated collision between enzyme and substrate will result in reaction, but it seems likely that all parts of the enzyme surface are not catalytically active. Moelwyn-Hughes (11) lets the probability that a substrate molecule hits the enzyme on an active spot be

$$\frac{\pi r^2}{\frac{4}{3}\pi R^2} = \frac{3}{4}\frac{r^2}{R^2}$$

where the numerator represents the area swept out by a spherical substrate molecule and the denominator is the relevant surface of the enzyme. When the radii of catalase and of H_2O_2 are substituted in this formula, the probability becomes 4.2×10^{-3}. Since it is quite clear that the 4 Fe atoms in catalase are intimately concerned with the catalytic action of catalase (18), the Fe might be considered the "active spot." In this case the probability that the collision between enzyme and substrate will involve an active spot might be given by the ratio of the volume of the Fe atoms in the catalase to the volume of the whole catalase molecule. This ratio is 5.2×10^{-3}, and is of the same order of magnitude as the probability obtained by Moelwyn-Hughes' method. When the theoretical rate calculated from the collision theory is multiplied by the probability that an activating collision will involve a catalytically active spot (composed of Fe?) on the catalase, the theoretical rate is only about 60 times greater than the experimental rate. Since about 29 per cent of all collisions may involve an active spot which is already occupied by a peroxide molecule (6), this difference is further reduced by this correction to a factor of about 37.

It is instructive to compare the rate at which catalase acts on H_2O_2 with the rate of other reactions involving heme proteins. Chance (3) reported an over-all rate for the peroxidase-H_2O_2-leucomalachite green system of 3.0×10^5 liter mole^{-1} sec.$^{-1}$, which is almost identical with the value of 3.4×10^5 liter mole^{-1} sec.$^{-1}$ found for the catalase-H_2O_2 system. From calculations by Haldane's method (6) (in which the Michaelis constant of 0.025 M is used) the minimum rate at which catalase combines with H_2O_2 is 4.75×10^7 liter mole $^{-1}$ sec. $^{-1}$. This compares closely with the rate of union of peroxidase with H_2O_2 of 1×10^7 liter mole^{-1} sec.$^{-1}$ (3) and of muscle hemoglobin with oxygen of 1.9×10^7 liter mole^{-1} sec.$^{-1}$ (8). Such results indicate a general similarity in the mechanism of reaction between heme proteins and small molecules.

It is a pleasure to acknowledge the assistance of Dr. Herbert Jaffe, who prepared the catalase, and Mr. Andrew Peacock, who performed part of the manometric experiments.

SUMMARY

The effects of temperature upon the crystalline catalase-H_2O_2 system have been studied over the temperature range from 2–68°. Below the optimum temperature of about 53° the rate of the enzyme-catalyzed reaction increases with temperature, in accordance with the Arrhenius equation; the activation energy is 4200 calories per mole; the entropy of activation is -23 calories per degree per mole.

Above the optimum temperature the enzyme-catalyzed reaction appears to decrease in rate with temperature, owing to the sharp increase in rate of enzyme inactivation with temperature. Catalase inactivation as a func-

tion of temperature between 57–68° has also been studied in the absence of substrate. By either method the activation energy for the heat inactivation of catalase below 62° is found to be 55,000 calories, and the entropy of activation is +90 calories. Between 62–68° the corresponding values are 255,000 and +690 calories for the energy and entropy of activation, respectively, of heat inactivation of catalase. Similarities are pointed out between heat inactivation of catalase and heat denaturation of proteins.

Theoretical rates of the enzyme-catalyzed reaction calculated on the assumption that catalase is a wall against which peroxide molecules collide, or calculated on the assumption that the collision theory applies, are about 10^4 greater than the experimental rate. Assumptions that activating collisions are effective only if they involve the active spot (Fe?) on the enzyme suggest that only 1 in 14,000 collisions is so oriented as to involve the active spot on the enzyme. Differences between theoretical and experimental values for the rate of the reaction could be largely accounted for if it is assumed that reaction results only from a properly oriented, activated collision. That a high degree of orientation is required for reaction is similarly indicated by the high negative value for the entropy of activation.

Similarities are pointed out between the kinetics of heme proteins such as catalase, peroxidase, and muscle hemoglobin.

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CLAVACIN, AN ANTIBIOTIC SUBSTANCE FROM ASPERGILLUS CLAVATUS*

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(Received for publication, May 2, 1944)

In 1942 Wiesner (1) and Waksman, Horning, and Spencer (2) demonstrated that filtrates from cultures of *Aspergillus clavatus* grown upon synthetic media possess antibiotic activity. These investigators extracted and partially purified the active material (which was named clavacin by Waksman) but did not report its isolation in pure form. As a result of the observations made by Waksman's group, particularly those on the antibiotic activity toward Gram-negative organisms, we became interested in this antibiotic substance and undertook the investigations of it as part of our work under Contract OEMcmr-155.

In the latter part of last year, Raistrick *et al.* (3) published a report on the isolation and characterization of patulin, an antibiotic substance produced by *Penicillium patulum* Bainier and, furthermore, showed that the same compound is produced by *P. expansum* (4). Subsequent to Raistrick's first report, Bergel *et al.* (5) and Hooper *et al.* (6) announced the isolation of a crystalline compound from *Aspergillus clavatus* which on the basis of the data submitted appears to be identical with patulin. Furthermore, Florey, Jennings, and Philpot (7) showed that the same compound is produced by *A. giganteus* Wehm, and Bergel *et al.* (5) and Chain, Florey, and Jennings (8) showed that the compound, claviformin, which the latter had previously isolated from *P. claviforme* in 1942 (9) is likewise identical with patulin. Although we had isolated a crystalline antibiotic from *A. clavatus* and prepared the semicarbazone, thiosemicarbazone, 2,4-dinitrophenylhydrazone, and oxime derivatives and a catalytic reduction product before the appearance of Raistrick's paper on patulin (3), it is obvious that the portion of this paper dealing with the characterization of the compound[1] can only be regarded as a confirmation of the earlier reports and for that reason is given in an abbreviated form. The other sections of this paper deal with the method of production, isolation of crystalline clavacin, ultraviolet absorption behavior, and studies on toxicity, all of which were conducted independently in this laboratory.

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and St. Louis University.

Progress of our work was impeded by the formation of a highly insoluble product differing in composition from crystalline clavacin during the process of recrystallization. Our analytical figures on the first crystalline product seemed to indicate the formula $C_9H_8O_5$ for clavacin but the analysis of the derivatives agreed much better with the formula $C_7H_6O_4$. The determination of molecular weight was difficult on account of the insolubility of clavacin in reagents commonly used in this laboratory in the Rast procedure, but when finally accomplished with tribromophenol as solvent gave a result which indicated the correct value to be $C_7H_6O_4$, the formula ascribed to patulin by Raistrick *et al.* (3).

Method of Production of Clavacin

In the production of clavacin by suitable cultures of *Aspergillus clavatus*[2] Wiesner (1) employed the Czapek-Dox medium, whereas Waksman (2) found the glucose-nitrate medium as modified by Clutterbuck *et al.* (10) to be satisfactory. It contains $NaNO_3$ 3.0, KH_2PO_4 1.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.01, and glucose (C.P.) 40 gm. in 1 liter of tap water. We have found that the addition of 10 gm. of Difco yeast extract or 2 ml. of corn steep liquor[3] per liter to the glucose-nitrate medium improved the yield of active substance. Under the conditions of our experiments the corn steep liquor was more effective than the yeast extract and accordingly we have routinely incorporated the former in the culture medium.

The reaction of the sterilized medium varied between pH 4.0 and 4.5. Glass baking dishes (12 × 7.5 × 2 inches) equipped with glass covers were used as culture vessels. About 600 ml. of sterile medium were added to each dish.

The character of the inoculum (*Aspergillus clavatus*) seemed to be one of the most critical factors in the development of an active culture fluid. It has been our experience on several occasions that a stock culture routinely propagated in the laboratory may lose much of its previously manifested ability to produce the antibiotic substance. Substrains originating from colonies which demonstrated maximal zones of inhibition on plates seeded with *Escherichia coli* or *Staphylococcus aureus* were found to be superior to the original strain in the production of clavacin. Consequently, such artificially selected substrains were employed in routine production.

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[1] However, before discontinuing our work on the structure of clavacin, we considered it important to ascertain that our product is indeed identical with patulin. Consequently, we prepared the phenylhydrazone and acetate, the iodo acid, $C_6H_9O_3I$, and in addition repeated the hydrolysis of clavacin with sodium hydroxide and sulfuric acid. In each instance, our data agreed with the data reported by Raistrick, which together with the data already obtained by us leave no doubt about the identity of our product and patulin.

[2] We are indebted to Dr. S. A. Waksman for a stock strain of this mold.

[3] We are indebted to Anheuser-Busch, Inc., for a supply of this material.

The inoculum consisted of a finely divided suspension of a 7 day culture mat obviously containing sporulating as well as vegetative elements of the plant. In preliminary experiments it was found that greater yields were obtained when the inoculum contained these vegetative elements. Tray cultures prepared in the manner described were incubated at 23–25° for periods varying from 5 to 11 days and the cultures harvested when maximal potency as determined by assay had developed.

Assay of Clavacin Samples

The method used in testing for potency consisted of the determination of the smallest amount of the antibiotic substance which would inhibit the multiplication of a 1:1000 dilution of a 24 hour nutrient broth culture of *Escherichia coli* added to an equal volume of a culture medium containing 1 per cent peptone and 0.5 per cent sodium chloride. Suitable serial dilutions of the dissolved sample were incorporated in standard portions of the medium and in turn inoculated with the diluted culture of *Escherichia coli*. These tubes, including suitable controls, were incubated at 37° for 18 hours, and then inspected for visual evidence of bacterial growth. Potency of the sample was recorded as the highest dilution of the substance which prevented growth under these conditions. Accordingly, our unit may be defined as the minimal amount of material necessary to inhibit growth completely in 1 ml. of the assay medium. Check assays with a standard clavacin preparation carried out each day gave surprisingly consistent results.

Method of Preparation

The culture medium was placed in the cold room as soon as it was harvested because at 5° the activity is retained for at least 2 weeks. The chilled fluid, after filtration to remove suspended material, was poured on a column of norit which readily adsorbed the active principle (1, 2). The pH of the medium usually varied between 5.5 and 6.0, which was found to be satisfactory for the adsorption. The adsorbent used, norit A, 20 to 50 mesh, which had been washed with dilute HCl and H_2O and dried, was coarse enough to permit rapid percolation. The harvested medium was run through the column as long as activity was removed and that portion of the filtrate which retained active material was then passed through a second column, thus avoiding any loss. The presence of activity in the filtrate was always preceded by the appearance of color. From 3 to 10 million units were adsorbed per kilo of norit, depending upon the potency of the medium, the capacity being greater for media of higher potency. The column was then flushed out with distilled water, drained as dry as possible by suction, and the norit spread on filter paper to dry at room temperature.

It was found that acetone and chloroform were best suited for eluting the active principle from the dry norit, while ethyl ether and ethanol were relatively ineffective. Extraction in an apparatus of the Soxhlet type was necessary, since even repeated extraction with cold solvent did not give complete recovery. After 10 to 20 hours of extraction usually more than 90 per cent of the activity had been eluted, although in a few instances recoveries as low as 60 per cent were encountered. Although extraction was generally more effective with acetone than with chloroform, particularly for the batches of norit from medium having a low potency, acetone removed much larger quantities of inert material, necessitating considerable purification before the crystalline material could be obtained.

Chloroform was satisfactory for extracting the active material from the norit used for media having a high potency. In such cases it was only necessary to concentrate the extract in order to obtain the crystalline compound.

Purification

The solution which was obtained by extraction of the norit with acetone was concentrated under reduced pressure to a thick oil which was then dissolved in the minimal volume of warm ethanol. This solution, upon the addition of 5 to 10 volumes of acetone and chilling in a bath of dry ice in cellosolve, yielded a copious precipitate which was inert. The supernatant solution was evaporated and the treatment was repeated with a larger proportion of acetone to alcohol. This process was repeated until precipitation no longer resulted and then the solution was concentrated to a small volume and 5 to 10 volumes of ethyl ether were added. The red oil which precipitated on chilling this mixture was relatively inactive. Additional amounts of the red oil were obtained by evaporating the supernatant solution and repeating the procedure with larger volumes of ether. The ether and acetone were finally evaporated under reduced pressure to assure the complete removal of the solvents, and the residue was taken up in warm dry chloroform. Any insoluble material was leached several times with small volumes of warm chloroform.

The chloroform solution was then fractionated by means of a permutit column. From 0.5 to 7 gm. of crude material in 10 to 50 ml. of chloroform was used for columns of permutit ranging in size from $\frac{3}{8}$ × 10 inches to 1 × 20 inches. The active principle could often be obtained by washing the column with chloroform but in some instances the addition of 0.25 to 0.5 per cent by volume of ethanol to the chloroform was required. The recovery was in most instances quantitative (see Table I). The potency of the less active fractions was substantially increased by a repetition of this procedure. The active principle was readily crystallized from the more active fractions by concentrating the solvent and allowing the concentrate to stand in the cold room.

Invariably on repeated recrystallization of these products from ether, benzene, chloroform, alcohol, or acetone the crystalline material eventually changed into an amorphous product which was insoluble in all of the above solvents as well as in water and glacial acetic acid. It was soluble in aqueous alkaline solution with immediate formation of a red color.

Properties of Clavacin

Clavacin crystallizes readily from chloroform, ether, or benzene to form colorless prisms melting at 109–110° (uncorrected) and is active in a dilution of 1:200,000 to 1:240,000 by the assay procedure which has been described previously. Analysis, C 54.58, H 4.28 per cent; theory for $C_7H_6O_4$, C 54.54, H 3.90 per cent. Molecular weight by the Rast procedure, with tribromophenol as solvent, 169; theory for $C_7H_6O_4$, 154. Analysis of the insoluble product (see the preceding paragraph) obtained from one of our best preparations gave C 51.72 and H 4.54 per cent.

Clavacin gives the same distribution coefficient between ether and H_2O as that reported by Raistrick (3) for patulin and is readily extracted from aqueous solution with butanol. It is not steam-volatile but sublimes at 70° under a high vacuum.

The ultraviolet absorption spectrum[4] of clavacin has a single maximum at λ 2760 A and the curve is the same for alcoholic and aqueous solutions. The $E_{1\,\text{cm}}^{1\%}$ values for most preparations which have been recrystallized sufficiently to raise the melting point to 108–110° fall between 900 and 950. However, upon additional recrystallizations this value usually drops below 900 and at the same time the biological activity diminishes. Active fractions having an $E_{1\,\text{cm.}}^{1\%}$ greater than 1400 may be obtained from permutit columns, but on repeated recrystallization the absorption decreases, as indicated above. There is no apparent loss of antibiotic activity, however, until the extinction coefficient falls below 900. In recrystallization it has been found in some instances that the extinction of the material in the mother liquor progressively increases, while that of the crystals decreases. Perhaps these changes are in some way related to the insoluble product which is formed when clavacin is repeatedly recrystallized, or perhaps the antibiotic substance produced by the mold is a labile compound having a greater extinction coefficient which passes into a more stable form during the process of purification.

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[4] A Beckman quartz spectrophotometer, manufactured by the National Technical Laboratories, South Pasadena, California, was used in this work.

TABLE I

Antibiotic and $E^{1\%}_{1\,\mathrm{cm.}}$ Values of Successive Fractions Removed by $CHCl_3$ from Column of Permutit

6.75 gm. of crude clavacin containing 600,000 units adsorbed on ¾ × 16 inch column.

Fraction No.	Volume	Solids	Units		$E^{1\%}_{1\,\mathrm{cm.}}$
			Total	Per mg.	
	ml.	*gm.*			
1	35	1.5	20,000	13	318
2	35	0.7	38,000	54	460
3	200	1.8	355,000	198	860
4	200	0.56	135,000	240	1450
5	150	0.13	27,000	208	1480
6	150	0.07	10,000	143	1040
Total ..		4.76	585,000		

TABLE II

Influence of Semicarbazone Formation on Biological Activity and Ultraviolet Absorption of Clavacin

200 mg. of clavacin dissolved in 10 ml of H_2O; 0.75 gm. of Na acetate and 0.5 gm. of semicarbazide HCl added.

Time of reaction	Treatment	Bioassay	$E^{1\%}_{1\,\mathrm{cm.}}$ at 2760 A
		units per mg.	
0	None	240	916
5 min.	Room temperature	160	796
35 "	Heated on water bath (red coloration)	40	338
80 "	" " " "	8	257
26 hrs.	Room temperature	8	108

Because of the tremendous advantages offered by spectroscopic analysis in regard to accuracy, speed, and specificity, we have used this procedure in our work on this and other problems of a related character. In spite of the irregular behavior of clavacin, ultraviolet absorption has been helpful in following the fractionation of the active material. This is illustrated in Table I which presents the data obtained from an experiment in which clavacin adsorbed on a permutit column was removed by washing with chloroform. This is also illustrated by the preparation of the semicarbazone (Table II) in which the absorption began to decrease immediately after the addition of the semicarbazide hydrochloride and sodium acetate to an aqueous solution of clavacin. The decreased absorption was paralleled by a diminished antibacterial activity.

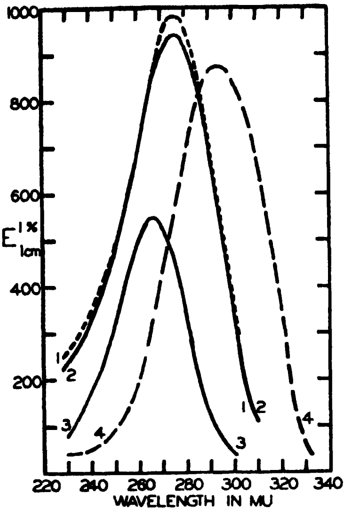

FIG. 1

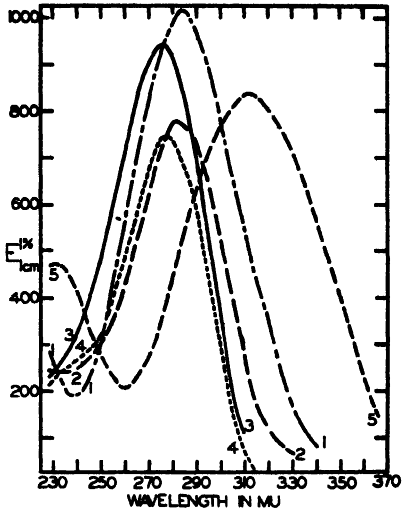

FIG. 2

FIG. 1. Absorption spectra. Curve 1, clavacin, peak at 2760 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 982, solvent 0 005 N alcoholic HCl. Curve 2, clavacin, peak at 2760 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 944, solvent alcohol. Curve 3, tetrahydro clavacin, peak at 2660 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 546, solvent alcohol. Curve 4, clavacin, peak at 2940 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 876, solvent 0.005 N alcoholic NaOH.

FIG. 2. Absorption spectra. Curve 1, clavacin oxime, peak at 2830 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 1020, solvent alcohol. Curve 2, amorphous insoluble product from clavacin, peak at 2800 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 780, solvent 0.05 N aqueous NaOH. Curve 3, clavacin, peak at 2760 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 944, solvent alcohol. Curve 4, clavacin acetate, peak at 2760 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 746, solvent alcohol. Curve 5, clavacin semicarbazone, peak at 3120 A, $E^{1\%}_{1\,\mathrm{cm.}}$ 837, solvent alcohol.

Figs. 1 and 2 show the ultraviolet absorption of clavacin under different conditions and that of some of its derivatives. The spectrum of an alcoholic solution of clavacin containing 10 γ per ml. remains unchanged after the solution has stood at room temperature in daylight for 3 months, while that of an aqueous solution of similar strength indicates that the compound has been almost completely destroyed.

Alkaline solutions of clavacin are highly colored. This color develops instantly upon the addition of alkali and disappears almost completely upon acidification. There is a shift of the maximum from λ 2760 A to λ 2940 A and an accompanying diminution of about 10 per cent in the extinction coefficient.

Chemistry of Clavacin

In this section we have recorded our chemical findings upon clavacin which confirm the reports of Bergel *et al.* (5) and Hooper *et al.* (6) that clavacin is identical with patulin which Raistrick *et al.* (3) have identified as anhydro-3-hydroxymethylene-γ-pyrone-2-carboxylic acid. The melting points and analyses of the derivatives of clavacin listed in Table III confirm the observations that similar derivatives of clavacin and of patulin are identical. Of these derivatives it was found that the acetate changes into an insoluble form upon repeated recrystallization.

TABLE III

Analyses of Derivatives of Clavacin

Derivative	M. p. (uncorrected)	Analysis, per cent						
		Found			Formula	Calculated		
		C	H	N		C	H	N
	°C							
Semicarbazone*	Decomposed	45.27	4.22	19.40	$C_8H_{11}O_4N_3$	45.50	4.27	19.71
Oxime†	155–156	49.66	4.03	8.07	$C_7H_7O_4N$	49.70	4.14	8.28
Phenylhydrazone‡	146	63.47	4.81	11.00	$C_{13}H_{12}O_3N_2$	63.93	4.94	11.49
Dinitrophenyl-hydrazone‡	Decomposed	46.46	3.41	16.51	$C_{13}H_{10}O_7N_4$	46.70	2.99	16.76
Acetate‡	115	55.03	4.25		$C_9H_8O_5$	55.18	4.08	

* Previously reported by Hooper *et al.* (6).

† Previously reported by Bergel *et al.* (5).

‡ Previously reported by Raistrick (3), Bergel (5), and Hooper (6) and their associates.

In agreement with the formation of a monoacetate, the determination of hydroxyl groups by the method of Peterson and West (11) indicates that one hydroxyl group is present per mole of clavacin. Treatment of clavacin with 20 per cent nitric acid yields oxalic acid; with sodium hydroxide and iodine iodoform is produced, a reaction known to be given by pyrones.

Oxidation with ammoniacal silver oxide, hydrolysis with sulfuric acid or sodium hydroxide, and the reaction with hydriodic acid show that the behavior of clavacin is the same as that found for patulin by Raistrick *et al.* (3). The iodo acid obtained by refluxing clavacin with concentrated hydriodic acid has a melting point of 93–94° (uncorrected) and analysis gave C 28.41, H 3.85 per cent. The calculated values for the γ-keto-ϵ-iodo-*n*-hexanoic acid obtained from patulin by Raistrick are C 28.15, H 3.54 per cent.

In aqueous solution, each molecule of clavacin adds 2 molecules of hydrogen in the presence of Adams' platinum oxide catalyst. The green color which develops at first disappears during the course of the hydrogenation, which is complete in 3 hours. The colorless hydrogenated product, after high vacuum sublimation, crystallizes from ether in white needles which melt at 111.5–112° (uncorrected) and gives C 53.37, H 6.0. The calculated values for $C_7H_{10}O_4$ are C 53.16, H 6.32.

Although Raistrick (3) found that catalytic hydrogenation of patulin in ethanol with the Adams platinum oxide catalyst caused the uptake of 2 molecules per molecule of patulin, our experience has been that 3.1 molecules of hydrogen are taken up per molecule of clavacin under these conditions. The same color changes are observed as are found in the hydrogenation in aqueous solution and the product obtained is a colorless oil. We were unable to crystallize or to prepare derivatives of it. Quantitative acetylation (11) indicated that no hydroxyl groups are present.

Mode of Action of Clavacin

The studies which we have made pertaining to the mode of action of this antibiotic appear to conform with the findings of other investigators. Crystalline clavacin is active against Gram-positive and Gram-negative bacteria, as has been reported by Waksman *et al.* (2) for less highly purified clavacin, by Chain, Florey, and Jennings (9) for claviformin, and by Raistrick *et al.* (3) for patulin. Clavacin exhibits bactericidal or bacteriostatic effects, depending upon the concentration. The crystalline material possesses considerable activity against the species of the *fungi imperfecti* tested, namely, *Rhizopus nigricans*, two strains of *Monilia albicans*, two strains of *Saccharomyces cerevisiae*, and *Sporotrichium schenkii*. In this connection, we found that clavacin is not autoinhibiting; that is, it does not interfere with the growth of *Aspergillus clavatus* when present in a concentration of 1 mg. per ml. of culture fluid. Limited antifungal activity (species not stated) was reported by Waksman *et al.* (2) for clavacin, while Anslow *et al.* (4) found that patulin exerted a powerful inhibitory effect on the growth of several species of *Pythium* including *Pythium debaryanum*.

Our experiments indicate that the activity of clavacin is not diminished by fresh human, horse, guinea pig, rat, and sheep sera when these are present in the culture medium in a concentration of 10 per cent. Likewise it is not inhibited by stored samples of beef and hog sera. On the other hand, it is completely (>90 per cent) inhibited by fresh rabbit serum. This inhibitory action is destroyed by heating the serum to 56° for 30 minutes or by standing in the refrigerator for several days. Raistrick *et al.* (3) found that patulin is not affected by human serum, while Chain *et al.* (9) state that such treatment (type of serum not stated) partially inactivates claviformin. Perhaps the explanation of this discrepancy lies in the nature of the sera used.

Toxicity of Clavacin

Although Waksman (2), Raistrick (3), and Chain (9) have observed the toxic nature of this antibiotic substance, we have attempted to obtain a more complete picture of its effects in the animal body. From the study of a group of over 100 mice, weighing 20 to 25 gm., it was determined that 0.2 mg. of clavacin given subcutaneously causes the death of approximately 50 per cent of the animals. For a smaller series of rats weighing about 200 gm., this effect was produced by approximately 5.0 mg. Paramecia and tropical fish (guppies) are killed in dilutions of 1:1,000,000 and 1:100,000, respectively. Clavacin acetate in benne oil injected subcutaneously into mice is about one-half as toxic as clavacin under the same conditions.

In mice clavacin is most toxic (about 0.10 mg.) when given intraperitoneally, and least toxic (about 0.75 mg.) when given orally. The injection of 0.5 to 0.7 mg. of clavacin by way of the tail vein was not lethal. The rate of absorption of clavacin is very rapid, as both rats and mice manifest profound effects within 5 minutes following the subcutaneous injection of an aqueous solution and mice show signs of sickness in 10 or 15 minutes after the material is administered in oil.

The general reactions to clavacin are about the same in both mice and rats, but may be observed more easily in the latter. Within 5 minutes after the injection of 5 mg. rats become restless and show increased activity, the breathing is heavy and labored, and fluid runs from the eyes and nose, causing a repeated washing reflex and shaking of the head. As time passes they become less active but continue to be restless, changing position frequently, and showing obvious signs of discomfort.

Edema of the subcutaneous tissues over the thorax is quite pronounced and at times extends to most of the subcutaneous tissues. In the rat, treated with the larger doses, fluid is found in the abdominal and thoracic cavities and edema of the lungs is so pronounced that at death they no longer float in 10 per cent formalin. Histological sections show considerable edema, congestion of the capillaries, and infiltration by leucocytes. In one extreme case the slide scarcely resembled lung tissues, the alveoli being filled with fluid and many leucocytes, but there was only a slight degree of hemorrhage.

At death the lungs and intestine frequently are deep red, the hyperemia apparently being caused partly by the concentration of the blood due to the edema and partly by the dilatation of the capillaries. While hemorrhage in liver and intestine is not observed and color changes in the liver are less apparent, nevertheless under the microscope the capillaries show considerable congestion. The edema and hyperemia induced by the administration of clavacin agree with the observations made by Raistrick *et al.* (3) following the injection of patulin.

In rats treated with clavacin there is a marked suppression of urine formation. Nine rats weighing about 200 gm. given subcutaneous injections of 5.0 mg. of clavacin voided little or no urine over periods up to about 15 hours. During this time the administration of 25 ml. of normal saline by stomach tube and intraperitoneal injection enhanced the edema but did not promote the production of urine to any appreciable extent. Control animals treated in the same way showed a marked increase in urinary excretion. After 24 hours the animals treated with clavacin and saline had voided only small quantities of urine, while the controls had excreted most of the fluid administered. Sections of the kidney revealed only slight congestion of the capillaries, evident but not extensive degeneration of the tubules, and slight hemorrhage.

Following the injections, necrosis usually develops at the site of injection, degenerative changes becoming apparent within 24 hours. In mice, thickening and hardening of the skin appear in 2 to 4 days, followed by open lesions in 4 to 6 days after the injection. When intravenous injections are made in mice by way of the tail, some fluid is usually lost into the subcutaneous tissues. Edema and damage to the tail are apparent within 24 hours and the distal portion usually drops off in a few days.

The intravenous injection of 25 mg. of clavacin into 7 kilo male dogs caused a transitory stopping of the heart, immediately followed by resumption at a markedly subnormal rate and then a gradual increase in heart rate and carotid blood pressure to a value above normal, simulating a typical vagal effect. Changes in respiration indicated a transient depression of the respiratory center. The gradual but continual decline in carotid pressure following the initial changes and the progressive increase in heart and respiratory rate together with the findings at autopsy indicated that fluid was slowly but continually being lost from the circulatory system.

In a similar experiment the injection of 10 mg. of clavacin did not cause a change in the carotid blood pressure within a period of 4 hours. In our experiments with dogs we did not observe the initial rise in blood pressure which was found by Chain, Florey, and Jennings (9) when patulin was injected intravenously into a cat anesthetized with chloralose.

SUMMARY

Methods for the production, preparation, and purification of clavacin, an antibiotic substance produced by *Aspergillus clavatus*, are described.

Analytical data and the chemical and physical properties of the pure crystalline material and some of its derivatives are presented. These findings confirm the identity of clavacin and patulin.

Clavacin is active against Gram-negative and Gram-positive bacteria and fungi. It is inhibited by fresh rabbit serum but not by the sera of the other species tested.

A preliminary toxicological study of this antibiotic substance has been made. In our hands it appears that this substance is highly toxic in the animal organisms.

We are indebted to the Theelin Fund administered by the Committee on Grants for Research of St. Louis University for financial support, to Mr. Francis A. Jacobs for his assistance in conducting the experiments on dogs, and to Mr. Joseph Yglesias for his helpful assistance in the experiments on fractionation and isolation of clavacin.

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REGENERATION OF HEAT-INACTIVATED PEROXIDASE*

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(Received for publication, April 14, 1944)

One of the old criteria of the presence of an enzyme is its lability toward heat. It must not be forgotten, however, that some enzymes found to be inactive after being heated to temperatures approaching 100° later recover an appreciable portion of their original activity on standing at a lower temperature.

The reappearance of potential activity after heating has been studied on pepsinogen by Herriott (1) and on trypsin by Northrop (2) who applied this property to the purification of the latter enzyme. The relation of such behavior to protein denaturation is evident from the studies of Anson and Mirsky (3) on hemoglobin and the most general opinion today is that the return of activity observed upon cooling heated enzyme solutions is due to a reversion of the denatured enzyme protein to its native state. Numerous less spectacular examples of such reversion may be cited. Probably the oldest and most extensive series of these deals with peroxidase.

Peroxidase is today frequently used in industry as a test substance to determine whether or not vegetables have been heated sufficiently in blanching before dehydration. Since dehydration is a matter of time, it has been suggested (4) that reversion of the denatured enzyme might take place and so produce positive tests in material that would have been negative if tested immediately. More information on the behavior of peroxidase thus appears to be desirable.

The first recorded observation of the regeneration of peroxidase appears to be that of Woods (5) who in 1901 observed that the peroxidase of tobacco leaves reappeared some time after inactivation by heating. Observations by Kulpsohn (6) on radishes (1908), by Deleano (7) on castor bean seedlings (1909), by Gramenitski (8) on an impure diastase preparation (1910), and by Biedermann and Jernakoff (9) on potato extracts led to similar findings. In 1924 Gallagher (10) found that preparations of mangold peroxidase gave tests for aldehyde and iron and showed that some aldehydes in the presence of iron gave rise to peroxidase-like activity. According to Gallagher, heating peroxidase produces a zymogen that contains aldehyde groups, and the regenerated enzyme is produced by the "catalytic action of iron."

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* Enzyme Research Laboratory Contribution No. 93. This research was supported in part by Bankhead-Jones funds. Part of the report is from a thesis submitted to Georgetown University by Sigmund Schwimmer in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Bach and Oparin (11) found that peroxidase regeneration did not occur in the absence of oxygen and confirmed the necessity for the presence of an iron compound. The iron-containing pigment which they concentrated was capable of initiating regeneration in a heat-inactivated solution of the enzyme. In the light of later findings by Kuhn *et al.* (12) on the constitution of peroxidase, it is likely that this "breathing pigment" was hematin. Kulikow and Babkowa (13) have suggested that regeneration is due to the elution of an adsorbed (and thereby heat-resistant) enzyme from the colloidal matter present. Pronin (14) and Bach and Wilensky (15) found the pH optimum for regeneration at neutrality. The latter workers used a highly purified enzyme prepared by ultrafiltration.

Older studies on enzyme action usually assume that only one enzyme is responsible for the generic reaction (peptide hydrolysis, oxygen liberation, etc.) under observation. Eventually a multiplicity of specifically acting enzymes is found, each circumscribed in its action to a limited number of substrates capable of undergoing the generic reaction. Recently two distinct peroxidases have been isolated by Theorell (16) from horseradish. Each enzyme was shown to consist of hematin groups attached to a specific protein. Gjessing and Sumner (17) have enlarged this work and have recently (18) described some of the divergent properties of milkweed and horseradish peroxidases.

The present paper compares the activity of peroxidase in plant juices from several sources by two well known methods. It was found that enzyme preparations from various sources differ greatly in the ratio of power to oxidize pyrogallol and to oxidize iodide. This ratio, although altered by purification, appears to be characteristic of the crude enzyme from a given source. It is not greatly changed when the activity of a heated preparation returns after cooling. During such heating a precipitate occurs in the system and may be centrifuged out. The precipitate remains inactive after cooling unless the supernatant liquid is again mixed with it; thereafter regeneration of the enzyme occurs in the mixture, often up to 35 per cent of the original activity. When the precipitate from cabbage juice is mixed with the supernatant from turnip juice, or *vice versa,* it is evident that the precipitate and not the liquid portion carries with it the characteristic that determines the ratio in question. These facts fit the theory that peroxidase is broken down by heat into an insoluble protein portion characteristic of the specificity and a soluble non-specific group. On cooling, these recombine to form peroxidase. The similarity of peroxidase proteins is obviously greater in behavior towards heat than in enzyme specificity.

EXPERIMENTAL

Preparation of Vegetable Juice—The vegetable was ground several times in a meat grinder, and the juice was squeezed out through cheese-cloth. 1 gm. of a purified diatomaceous filter aid was added for each 100 ml. of juice and the suspension was filtered after 10 minutes through a Buchner funnel containing a layer of filter aid. From both turnips and cabbage a clear yellow liquid was obtained which proved to be stable under toluene for 2 months at 6°.

Technique for Regeneration—A 2 ml. sample of clarified juice of known activity was pipetted into each of six test-tubes (15 × 150 mm.). The tubes were then placed in a water bath at the temperature and for the time desired. Thereafter they were put into an ice-salt mixture, and stirred vigorously. The contents of two such tubes were assayed at once; others, together with unheated controls, were kept at the regeneration temperature or otherwise treated according to the conditions of the experiment. The results are given as per cent of the original activity found after the treatment in question.

Determination of Peroxidase. Delayed Oxidation of Hydrogen Iodide—This method is a modification of the one introduced by Jayle (19) and adapted for field use by Davis (20). When peroxidase is placed in a buffered solution containing an excess of hydrogen iodide, starch, hydrogen peroxide, and a limited amount of some strong reducing agent (thiosulfate, ascorbic acid, ferrocyanide, etc.), a blue color ("flash reaction") will occur after an interval that depends upon the concentration of the peroxidase.

In the present experiments a dose of enzyme, always diluted to 9 ml., was added to 40 ml. of reaction mixture in a thermostat at 25°. The molar concentrations of the constituents of the reaction mixture were as follows: acetate buffer (pH 4.7) 0.02, $Na_2S_2O_3$ 0.001, KI 0.027. The concentration of boiled soluble starch in the mixture was 0.1 per cent. After addition of the enzyme solution 1 ml. of 0.9 per cent hydrogen peroxide solution was added to the mixture and the time required for the appearance of the blue color was measured.

1 unit of peroxidase activity[1] has been taken to be the amount of enzyme that will cause the liberation of I_2 (and therefore the appearance of the blue color) in 1 minute under the stated conditions.

Pyrogallol-Oxidizing Activity—The method of Balls and Hale (21) was used. For convenience 1 unit has been defined as the amount of enzyme that will oxidize, within 1 minute under specified conditions of reaction, a quantity of pyrogallol (contained in 25 ml. of reaction mixture) equivalent to a decrease in titration of 1 ml. of 0.01 N thiosulfate.

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[1] For purposes of making a ratio of the activity shown by this method to that measured by the oxidation of pyrogallol, it is necessary to express the results in units. Such units need only be numbers that are directly proportional to the quantity of enzyme, as determined under the conditions employed. The simpler the derivation of such numbers the better they are for this purpose. It is not claimed that they will be found generally applicable.

Results

Effect of Time and Temperature of Heating—Table I shows the values for peroxidase (iodide method) found in the samples of turnip juice heated at different temperatures and for various periods, then allowed to stand at 25° or at 6° to regenerate the enzyme. Inactivation was of course more rapid when the enzyme was heated at the higher temperatures, but enzyme inactivated by brief exposure at a high temperature reverted more completely to the active form on cooling than did enzyme inactivated to the same extent by a longer exposure at a lower temperature. This effect was illustrated further in an experiment in which 0.2 ml. of the juice was heated to 105° for 30 seconds. Immediately thereafter only 10 per cent of the original activity remained. This increased to 65 per cent during storage at 26° for 20 hours. In this case the speed of inactivation was the highest and the extent of regeneration the greatest observed in this series of tests.

TABLE I

Regeneration of Turnip Peroxidase

Heating temperature	Time of heating	Per cent of original activity		
		Immediately after heating	After 20 hrs. incubation at	
			25°	6°
°C.	*min.*			
	0.0		100	100
70	4	61	79	71
	8	55	70	63
	12	42	55	50
	60	23	30	27
80	2	45	71	57
	4	35	49	40
	8	30	39	36
90	1.0	37	60	50
	3.0	8	22	12
100	0.5	32	74	54
	1.0	12	44	
	1.5	4	30	15

From the data of Table I it appears that the shorter the time of exposure to heat has been, the greater is the portion of enzyme subsequently regenerated. The rate of destruction of the inactive enzyme by heat is presumably slower than that of the active enzyme. It is evident that when the permanent inactivation of peroxidase is desired it is not sufficient to heat the material merely until an immediate test on the product is negative, for a negative test, especially when obtained after brief heating at a relatively high temperature, may be followed after cooling by the reappearance of the enzyme.

Effect of Time and Temperature of Regeneration—In the same series of experiments (Table I), it may be seen that regeneration is not as complete at 6° as at 25°. Fig. 1 shows further that the regeneration is a time reaction, completed under the conditions of the experiment in about 4 hours.

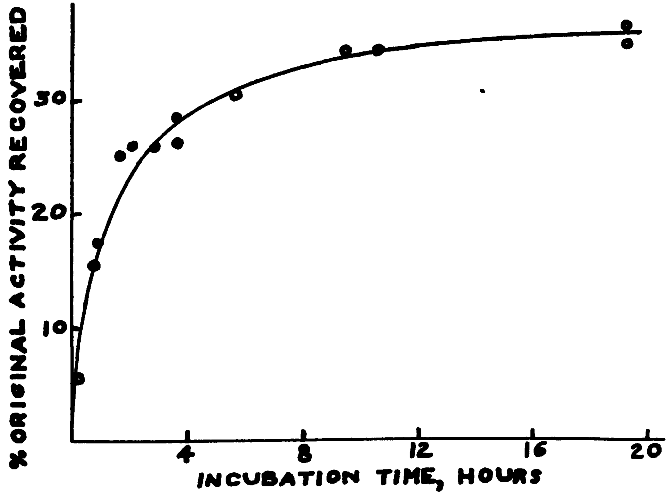

Fig. 1. Regeneration of heat-inactivated peroxidase

The results obtained at the lower temperature of regeneration, indicating that either equilibrium between native and denatured protein has not been reached or that the equilibrium is shifted away from regeneration of peroxidase, cannot be interpreted merely as a reversal of denaturation, for lowering of the temperature should shift the equilibrium towards regeneration.

Behavior of Juice on Heating—A slight precipitate which could be removed by centrifuging was seen to form when either cabbage or turnip juice was heated. Regeneration of the enzyme did not occur in the absence of this precipitate (Table II). When the system was cooled, the precipitate did not entirely dissolve, but all the regenerated activity was found in the supernatant. Regeneration of enzyme did not occur if fresh unheated juice was used instead of the supernatant fraction of the heated juice. Nor did it occur to any marked extent if a precipitate prepared by brief heating was mixed with a supernatant fraction prepared by longer heating. Furthermore, regeneration did not occur if the precipitate and supernatant were kept separate for about 5 hours and then recombined.

Ratio of Hydrogen Iodide to Pyrogallol-Oxidizing Activity—By the modified Jayle method and the Balls-Hale method used here two different types of peroxidase activity are measured, most probably two different enzymes, because the ratio of these activities may be varied by precipitation of the enzymes from the original juice with ammonium sulfate, but not by merely heating and cooling. The ratio of these two activities varies also with the source of the enzyme. For example, barley extracts are relatively rich in the pyrogallol-oxidizing factor, whereas cabbage juice is rich in what oxidizes hydrogen iodide. Turnip juice is midway between (Table III). These ratios thus furnish a way of distinguishing certain peroxidase preparations from one another.

TABLE II

Regeneration of Peroxidase Requires Both Precipitate and Supernatant

2 ml. samples heated for 1 minute at 100°; incubated at 26° for 20 hours; analysis by iodide method.

Treatment	Per cent of original activity		
	Before incubation	After incubation	Difference
Whole turnip juice .	13	51	38
Ppt. alone		3	
Supernatant alone	12.5	12.5	0
" (removed after incubation)		49	
Ppt. incubated with unheated juice	103	102	−1
" (prepared as usual) incubated with supernatant prepared from juice heated 3 min.	7	15	8
Ppt. (prepared as usual) incubated with supernatant prepared from juice heated 5 min.	4	12	8
Ppt. and supernatant incubated separately for 5 hrs., recombined, and then incubated for another 20 hrs...	13	18	5

It was found that turnip juice after being heated, and later allowed to stand at a lower temperature, gave a value of 1.13 for the ratio of pyrogallol-oxidizing factor to the other in the regenerated enzyme mixture (Table IV). Similarly, cabbage juice gave a value of 0.75. When turnip juice was centrifuged immediately after heating, and the precipitate was combined with the supernatant from similarly heated and centrifuged cabbage juice, the ratio between the two oxidizing factors approximated that in the original turnip juice. Cabbage precipitate and turnip supernatant, on the other hand, regenerated an enzyme containing these factors in a ratio approximated by that in cabbage juice.

TABLE III

Ratio of Pyrogallol and Iodide Units Varies for Different Material

Plant	Preparation	Ratio, units of pyrogallol / units of iodide
Cabbage	Undiluted clarified juice	0.50
Horseradish	Crude protein fraction	0.81
"	Partly purified proteins	1.2
Turnip	Undiluted clarified juice	1.1
Wheat	Water extract of whole wheat	2.2
Barley	" " " " grain	2.6
"	Proteins from water extract	3.2

TABLE IV

Effect of Interchanging Precipitate and Supernatant Liquid from Cabbage and Turnip Juice

The activity of iodide and pyrogallol is expressed in units per ml.

Sample	Treatment	Activity, iodide	Activity, pyrogallol	Ratio, units of pyrogallol / units of iodide
Cabbage juice	Before heating	2.14	1.25	0.58
	After "	0.23	0.23	1.00
	" incubation	0.97	0.79	0.81
	Activity regenerated	0.74	0.56	0.75
Turnip "	Before heating	2.20	2.34	1.06
	After "	0.30	0.24	0.80
	" incubation	1.08	1.08	1.00
	Activity regenerated	0.78	0.84	1.13
" ppt. + cabbage supernatant	Before incubation	0.23	0.26	1.12
	After "	0.85	0.96	1.12
	Activity regenerated	0.62	0.70	1.13
Turnip supernatant + cabbage ppt.	Before incubation	0.30	0.24	0.80
	After "	0.75	0.56	0.75
	Activity regenerated	0.45	0.32	0.71

DISCUSSION

It is evident that both the precipitate formed on heating and the liquid supernatant thereto contain factors essential for the reappearance of peroxidase. The precipitate, however, carries with it the specific characteristics of the enzyme that relate to the oxidation of pyrogallol or hydrogen iodide.

The common concept of peroxidase as a conjugated protein containing a porphyrin group offers an adequate basis for explaining the foregoing observations. The simplest assumption appears to be that three general changes (probably related, however) occur when peroxidase is heated; namely, denaturation of the enzyme protein, separation of the prosthetic group therefrom, and precipitation of the protein. Denaturation is a logical assumption in view of Anson's finding (22) that in the case of proteins of the hemoglobin type, in which the prosthetic group is firmly bound, this group can probably be separated only when the protein is denatured. Precipitation of the enzyme protein is an observed fact, though it may be due to inherent insolubility, or to adsorption on other proteins precipitated by heat. As no peroxidase whatever reappears in the supernatant fraction, it may be concluded that precipitation of the insoluble factor is practically complete.

The explanation of reactivation of the enzyme must take into account a reversal of all three changes, and there is thus no reason to suppose that the reappearance of the enzyme is dependent merely on the rate of reversion of the protein.[2]

SUMMARY

The regeneration of peroxidase as a function of time and temperature was studied. It was found that regeneration is largely a function of the heating rate, has a positive temperature coefficient, and is a time reaction. Factors that are essential for regeneration exist in both the precipitate formed upon heating and in the supernatant therefrom.

Evidence is given to show that vegetables contain more than one peroxidase. The different peroxidases vary in their activities towards iodide and pyrogallol. An untreated vegetable juice may be characterized by the determination of these activities. The precipitate formed when the juice is heated carries these characteristic properties with it.

Reappearance of the enzyme after heat treatment involves resolution of an insoluble component, recombination with a soluble group, and reversion of enzyme protein to its native state.

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[2] Since the presence of oxygen is required for regeneration (*cf.* Bach and Oparin (11)), it is likely that hematin, not heme, can undergo combination to form an enzymatically active molecule.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXVI. CONCERNING THE STRUCTURE OF TUBERCULOSTEARIC ACID*

By SIDNEY F. VELICK

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(Received for publication, April 13, 1944)

Tuberculostearic acid is the lowest member of the series of branched chain fatty acids isolated by Anderson and coworkers from the lipids of the tubercle bacillus (1). Its probable structure, 10-methylstearic acid, was advanced by Spielman on the basis of degradation studies and also from comparison of the purified acid with synthetic *dl*-10-methylstearic acid (2). The natural and synthetic acids were shown to yield apparently identical crystalline derivatives but the melting points of the free acids were 10° apart. This discrepancy was attributed to the fact that tuberculostearic acid, in view of its biological origin, was probably optically active. Although no optical activity could be observed, evidence was cited which indicated that the specific rotation of a structure of this type might be sufficiently low to escape detection.

In the present communication the results of an x-ray diffraction study of the amides of tuberculostearic acid and *dl*-10-methylstearic acid are presented. X-ray methods have proved of particular value in the study of the higher straight chain fatty acids from natural sources (3). There was reason for believing that if, as suggested, tuberculostearic acid were optically active its x-ray diffraction pattern would show certain slight differences from that of the racemic mixture. The alternative explanation for the difference in the melting points of the synthetic acid and the natural product was that tuberculostearic acid might contain a small amount of isomeric impurity (such as 9-methylstearic acid). This eventuality would likewise be expected to produce an observable effect on the x-ray diffraction pattern. Authentic samples of the *dl*-10-methylstearic acid and tuberculostearic acid were made available by Professor Anderson.

Powder photographs of tuberculostearic acid (m.p. 10°) taken at low temperatures by Spiegel-Adolf and Henny contained lines which could not be indexed (4) but which appear to correspond to lateral spacing of an extended chain. For the present experiments the higher melting amides were selected for study. These were prepared according to Spielman's procedure (2) and were obtained in the form of thin leaflets. The method

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* The present report is part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

of obtaining the diffraction photographs has been generally employed on the simpler long chain compounds. Thin aggregates of the leaflets were oriented by gentle pressure on 10 mm. glass cover-slips. X-ray reflections were obtained from the crystal planes parallel to the oriented surface by oscillation at small grazing angles in a beam of nickel-filtered copper radiation. A cylindrical camera of 51.2 mm. radius and a 100 mm. flat camera were employed. They were checked by diffraction measurements of crystals of known structure. The cylindrical films were measured under magnification with a vernier device and the spacings calculated from the Bragg equation $n\lambda = 2d \sin \Theta$. The relative intensities were estimated by visual comparison.

Both substances gave a series of sharp $00l$ reflections. Under the experimental conditions the presence of homologous impurities in crystals of long chain compounds causes the higher order $00l$ reflections to vanish (3). The lattice defects resulting from isomeric impurities would be expected to have the same effect. In view of the large number of reflection orders observed it is unlikely that tuberculostearamide contains any appreciable amount of homologous or positional isomeric impurity.

The (001) spacing of *dl*-10-methylstearamide was 39.1 A and that of tuberculostearamide 37.8 A. This difference persisted in samples recrystallized from dilute methanol and in preparations crystallized by slow cooling of the melt. It therefore could not be due to an accidental choice of different polymorphic modifications. The order of magnitude of the spacings indicates that the molecules crystallize in double layers in which the molecules are inclined toward the (001) planes. The difference in the spacings is equivalent to a 5° to 7° difference in the angle of inclination.

A slight but definite difference was likewise observed in the intensity distributions which are shown diagramatically in Fig. 1, *a* and *b*. It should be observed that the intensity relationships of the lower orders are identical in the two diagrams but that a slight difference becomes apparent in the sixth and seventh orders. The tenth and fourteenth orders are moderately strong in both diagrams and the twelth, fifteenth, and sixteenth orders weak. Differences are observable in the ninth, thirteenth, and seventeenth orders.

The intensities of the $00l$ reflections from crystals of this type are given by the expression

$$I_{00l} = K \frac{1 + \cos^2 2\Theta}{\sin 2\Theta} \left[\sum_n f_n \cos 2\pi l z_n \right]^2$$

where f_n is the scattering factor of the nth atom, z_n is its coordinate, l is the order of reflection, and Θ is the Bragg angle. The contribution of each atom to the intensity of the diffracted beam is given by a term ($f_n \cos 2\pi l z_n$). In the lower orders this function is relatively insensitive to small changes in z_n. This means that the intensities of the lower order reflections are determined chiefly by the broader features of the molecular and crystal structure such as the general position of the branch but not its exact level. As the order l increases, the cosine function becomes increasingly sensitive to small changes in z_n. Accordingly the higher orders are influenced by slight coordinate shifts such as might exist between the optically active isomer and the *dl* mixture.

The agreement of the lower order intensities indicates that the side chains in tuberculostearamide and in *dl*-10-methylstearamide occupy the same general position. That a slight difference in *average* position exists is indicated by the discrepancies in the intensities of the higher orders. Since it has been shown to be unlikely that appreciable amounts of isomeric impurity are present, the difference must be due as previously suggested to the optical activity of the natural product. The physical basis of the coordinate shift must be sought in the fact that the branch in the optically active compound projects from one side only of an asymmetric tilted chain, whereas in the racemic mixture it projects from either side. The resulting effect upon the packing of the molecules in the crystal is revealed by the differences in the long spacing.

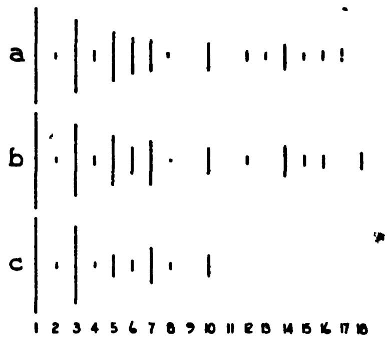

FIG. 1. Diagram of intensity distribution of 00l reflections. (*a*) *dl*-10-methylstearamide, (*b*) tuberculostearamide, (*c*) calculated for 10-methylstearamide.

The actual numerical evaluation of the intensity equation is of particular importance in locating the position of branches in unknown structures. It has been applied with some success to the simpler case of the normal primary alcohols by Wilson and Ott (5). To determine its applicability and limitations in the case of the fatty acids calculations were made for stearic acid and for 10-methylstearamide. The problem was to find by trial a set of coordinates consistent with the molecular and crystal structure, which when substituted in the intensity equation give an intensity distribution similar to the one obtained experimentally. The procedure followed was similar to that employed by Wilson and Ott (5).

2 molecules in adjacent layers are shown in general position in Fig. 2, *A*. The coordinates z_n are the projections of the atomic centers upon a line normal to the (001) planes, one of these planes being taken as origin. It can be seen that the coordinates are functions of the degree of rotation of the tilted zigzag chain about its long axis and also of the distances separating adjacent layers of molecules. The coordinates found by trial to give an approximately correct relative intensity distribution are included in the following working form of the intensity equation. They are expressed as fractions of the (001) spacing.

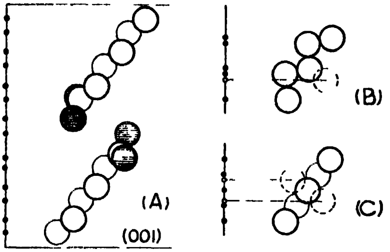

FIG. 2. (*A*) tilted molecules (shortened) in general position showing general relation of molecules in the double layer and the projection of the atomic centers. The shaded circles are oxygen atoms. (*B*) a carbon chain tilted at the same angle as in (*A*) but rotated about its axis, showing altered projection of atomic centers and the projection of a methyl side chain (broken line). (*C*) a carbon chain rotated in the second extreme position, showing evenly spaced projections of the carbon atoms. Two possible projections of a methyl branch, corresponding to *d* and *l* isomers, are shown.

$$\begin{aligned} I_{00l} = K\,\frac{1+\cos^2 2\Theta}{\sin 2\Theta}\,[&9(\cos 2\pi l\ 0.0261) + 8\{\cos 2\pi l\ 0.0522 \\ &+ \cos 2\pi l\ 0.0783 + \cos 2\pi l\ 0.1044 + \cos 2\pi l\ 0.1305 + \cos 2\pi l\ 0.1566 \\ &+ \cos 2\pi l\ 0.1827 + \cos 2\pi l\ 0.2088 + \cos 2\pi l\ 0.2349 + \cos 2\pi l\ 0.2610 \\ &+ \cos 2\pi l\ 0.2871 + \cos 2\pi l\ 0.3132 + \cos 2\pi l\ 0.3393 + \cos 2\pi l\ 0.3654 \\ &+ \cos 2\pi l\ 0.3915 + \cos 2\pi l\ 0.4176 + \cos 2\pi l\ 0.4437\} \\ &+ 14(\cos 2\pi l\ 0.4698) + 9(\cos 2\pi l\ 0.4911)]^2 \end{aligned}$$

The calculated and observed intensities are shown diagramatically in Fig. 3. Aside from small quantitative differences the agreement of the patterns is good through the eighteenth order. It should be noted that this simplified treatment does not account for the fact that for close packing alternate molecules may be rotated in opposite directions. Similarly the carboxyl groups, which may be rotated independently of the rest of the chain, may have a slightly different orientation from that indicated. The above coordinates must therefore be considered to represent average positions of atomic layers.

When an additional term ($9 \cos 2\pi l\, 0.2349$) corresponding to a secondary methyl group at the level of the 10th carbon atom is introduced into the summation, a new intensity distribution is obtained. The new distribution is shown in Fig. 1, *c*. There is seen to be a qualitative agreement with the $00l$ intensity distribution of tuberculostearamide through the tenth order. The agreement is sufficient to show that the arrangement of the molecules in the tuberculostearamide crystal is very similar to the arrangement in the stearic acid crystal, and provides independent proof of structure.

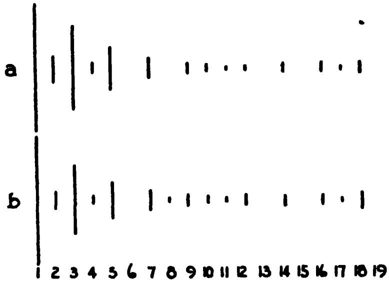

FIG. 3. Relative intensities of the $00l$ reflections from stearic acid crystals: (*a*) calculated, (*b*) observed.

Because of the number of variables and the approximate nature of the calculations and intensity measurements a further refinement of the coordinates to account quantitatively for the differences between tuberculostearamide and *dl*-10-methylstearamide did not seem warranted. The possibility of applying a similar treatment to other isomeric methylstearic acids will be discussed in a subsequent report.

SUMMARY

1. The (001) crystal spacing of tuberculostearamide is 37.8 A and of *dl*-10-methylstearamide 39.1 A.

2. The intensity distributions of the $00l$ reflections of the two compounds show differences in the higher orders.

3. The observed differences are consistent with the hypothesis that tuberculostearic acid, although showing no detectable optical rotation, is optically active.

4. Calculation of the $00l$ intensities from model structures indicates that

stearic acid and tuberculostearamide molecules have similar orientations in the crystal.

5. The results support the structure *d*- or *l*-10-methylstearic acid proposed for tuberculostearic acid.

Much of the x-ray equipment used in the present work was made available through the generosity of Dr. George Switzer of the Department of Geology and Dr. Charlton Cooksey of the Department of Physics of Yale University.

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LETTERS TO THE EDITORS

THE ACTION OF β-AMYLASE ON CORN GLYCOGEN*

Sirs:

In the original work[1] on corn glycogen, one of the criteria used for its identification was the rate of its destruction by malt amylase. No determination was made of the extent of this destruction when it was allowed to go as far as possible. Meyer and Press[2] have reported that by long continued action of β-amylase glycogen is about 45 per cent broken down, whereas starches are more strongly attacked, the destruction being 60 to 70 per cent for whole starches and 100 per cent for β-amylose.

In the light of this work, the extent of enzymatic destruction of corn glycogen[3] is being studied. The enzyme used is β-amylase prepared from wheat by the method of Ballou and Luck.[4] Under conditions similar to those of Meyer, corn glycogen was only about 20 per cent destroyed, whereas in the same experiments the destruction of rabbit liver glycogen was 45 per cent, and of potato and corn-starches 62 and 70 per cent respectively.

These results, interpreted in terms of Meyer's theories, imply that the corn glycogen has a more highly branched structure than has animal glycogen. Thus a series might be set up, of increasingly branched structure, β-amylose (with no branching), then amylopectin, animal glycogen, and corn glycogen.

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Received for publication, May 31, 1944

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* This work was aided by a grant from Mead Johnson and Company.

[1] Morris, D. L., and Morris, C. T., *J. Biol. Chem.*, **130**, 535 (1939).

[2] Meyer, K. H., and Press, J., *Helv. chim. acta*, **24**, 58 (1941).

[3] The name "phytoglycogen" suggested by Sumner and Somers (Sumner, J. B., and Somers, G. F., *Arch. Biochem.*, **4**, 7 (1944)) seems unecessary. It is not yet clear that corn glycogen differs from liver glycogen, let us say, any more than the latter does from oyster or yeast glycogen.

[4] Ballou, G. A., and Luck, J. M., *J. Biol. Chem.*, **139**, 233 (1941).

A PROTEOLYTIC INHIBITING SUBSTANCE IN THE EXTRACT FROM UNHEATED SOY BEAN MEAL

Sirs:

In studies upon the utilization of unheated soy beans by the chick, extracts made with dilute acid at pH 4.2 (the isoelectric point of most soy bean proteins[1]) were found to contain a substance which greatly retarded the activity of trypsin *in vitro*.

These extracts could be quite largely deproteinized by precipitation by kaolin without loss of activity of the proteolytic inhibitor. The substance could be further purified by precipitation of the supernatant liquid from the kaolin-treated extract with 60 per cent acetone and reextraction of this precipitate with water. Most of the proteolytic inhibition was destroyed, however, by precipitation of the kaolin-treated extract with 60 per cent ethyl alcohol. Likewise no inhibiting activity was found in the extracts from raw soy bean flake previously soaked in 45 per cent alcohol.

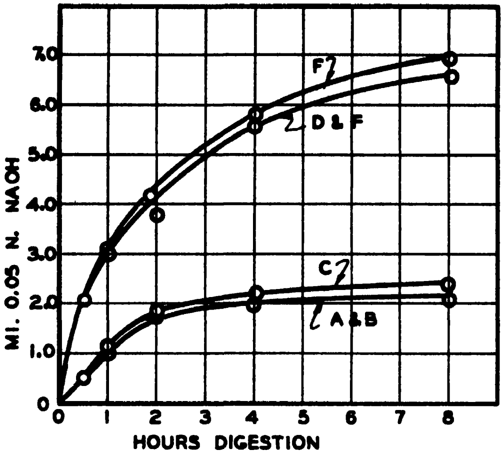

Fig. 1. Effect of treated extracts of uncooked soy bean meal upon tryptic digestion. Curve A, untreated dilute acid (pH 4.2) extract; Curve B, kaolin-treated dilute acid extract; Curve C, extract from acetone precipitate of dilute acid extract; Curve D, dialyzed, kaolin-treated dilute acid extract; Curve E, autoclaved dilute acid extract (not deproteinized); Curve F, no extract.

When 5 ml. of the dilute acid (pH 4.2) extract from uncooked soy beans or of the water extract of the acetone precipitate from the extract were added to a digestion mixture consisting of 50 ml. of 5 per cent soluble casein and 0.12 gm. of Armour's trypsin (1:50), the proteolytic action over an 8 hour period was inhibited, as shown in Fig. 1. The digestions were carried out at pH 8.0 and 37°. The hydrolysis was followed by means of the formol titration.

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[1] Circle, S. J., Thesis, University of Chicago (1941).

Activity of the proteolytic inhibitor was lost upon dialysis of the extracts and was destroyed by autoclaving either the soy bean meal or the extracts.

Evidence thus far indicates that the inhibitor secured by the precipitation with acetone and reextraction with water is quite unstable under ordinary conditions.

Previous work at the Nebraska Experiment Station, soon to be published, has shown that a factor causing growth retardation in chicks can be extracted from unheated soy beans with dilute acid (pH 4.2), leaving a residue which compares favorably in nutritive value with heated or autoclaved beans. The similarity in properties of the growth-retarding factor and the proteolytic inhibitor indicates that the two may be identical. Conclusive proof, however, has not been obtained with feeding trials, owing probably to the instability of the partially purified inhibitor used in the tests.

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Received for publication, June 3, 1944

THE STRUCTURE OF A NEW METABOLIC DERIVATIVE OF QUININE*

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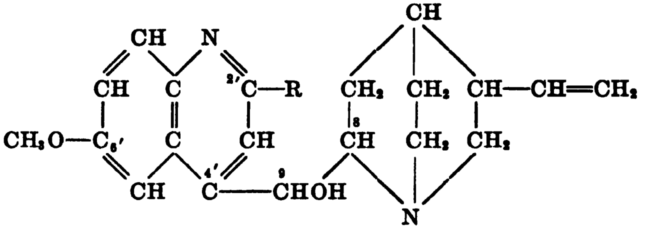
(Received for publication, March 13, 1944)

The isolation and crystallization of a product obtained by the *in vitro* action of rabbit liver on quinine (I) have recently been described by Kelsey, Geiling, Oldham, and Dearborn (1). The work reported here was undertaken with the object of obtaining information, as rapidly as possible, as to the possible structure of this product, which might prove useful in other malarial investigations. Thus no attempt has been made to obtain or present a rigid proof of structure of the metabolic product, which, for convenience, will be referred to as QDP (quinine-derived product). Evidence will be presented which suggests that QDP is levorotatory 2′-hydroxy-6′-methoxy-3-vinylruban-9-ol (II), according to the notation introduced by Rabe (2).



(I) R = H

(II) R = OH

EXPERIMENTAL

QDP is soluble in the alcohols, slightly soluble in acetone, ether, or chloroform, and insoluble in water and the hydrocarbon solvents. It exhibits a dark blue fluorescence in all its solutions and in contrast to quinine the fluorescence is not depressed by chloride ion.

QDP in chloroform solution was adsorbed on a column of calcium carbonate (Merck, heavy powder) and the chromatogram developed with chloroform containing 2 per cent ethanol. In ultraviolet light only a single fluorescing zone appeared on the column, which during further develop-

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* This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

† Contribution No. 958.

ment migrated slowly, without differentiation, into the chromatographic filtrate.

QDP crystallizes in long colorless needles when its methanol solution is diluted with water almost to the point of precipitation and is then allowed to stand in a desiccator over calcium chloride. In the melting block, the crystals show a distinct change in crystal structure at 150° (1), finally melting to a clear dark brown liquid at 247.5–248.5° (corrected). The chemical properties appeared unchanged by heating at 160° for several hours. QDP is optically active, a solution of 0.0165 gm. in 2 ml. of ethanol in a 2 dm. tube having a rotation of $-0.54°$; $[\alpha]_D^{25} = -65.5° \pm 0.5°$.

QDP is soluble in dilute mineral acids, from which it can be precipitated by dilute NH_4OH. It is insoluble in NH_4OH or in N alkali hydroxides but dissolves quite slowly, even on heating, in 2 to 3 N aqueous NaOH or KOH. When a suspension of QDP in 6 N NaOH or KOH is warmed, an insoluble oil is formed, which on dilution of the alkali to about 3 N readily dissolves. QDP cannot be extracted from a 3 N NaOH solution with a mixture of ether and butanol, but may be recovered unchanged, even after prolonged boiling, by acidification with dilute acid and neutralization of the acid solution with NH_4OH.

Despite this apparent acidity, QDP does not appear to have a titratable acid group and it does not react with diazomethane or give a color reaction with ferric chloride.

Analyses of QDP—A sample of QDP was purified for analysis by repeated recrystallization from methanol and water. The final sample was dried at 110° *in vacuo* but proved to be somewhat hygroscopic after drying. The individual samples were therefore dried to a constant weight and weighed separately in a closed system for analysis.

$C_{20}H_{24}O_3N_2$.	Calculated.	C 70.56,	H 7.11,	N 8 23,	MeO 9.11
340.4	Found.	" 70.28,	" 7.16,	" 8.51,	" 9.08
	"	" 70.41,	" 7.01,	" 8.53,	" 9.06
	"	" 70.40,	" 7.09,	" 8.32,	" 9.08

Molecular weight determinations by the Rast method proved unsatisfactory; the QDP sample darkened somewhat in the camphor melt (180°). The results obtained varied between 475 and 532.

Potentiometric Titration—50 ml. of a 90 per cent ethanol solution containing 4.4×10^{-4} mole of quinine and 50 ml. of a 90 per cent ethanol solution containing 4.3×10^{-4} mole of QDP were titrated with 0.0466 N HCl. The pH measurements were made with a Beckman pH meter after each addition of 1 to 2 ml. of acid. The graphical point from a plot of pH against the volume of acid added which corresponded to half neutralization of the first basic group was taken as the ionization constant, K_{b_1}; this point for both quinine and QDP was pH 7.45.

At the calculated half neutralization point of the second basic group of quinine, 14.15 ml. of acid had been added and the observed pH was 4.27. At the same point for QDP, 13.85 ml. of acid had been added and the pH was 2.80. In the calculation of the constant K_{b_2} for the second basic group

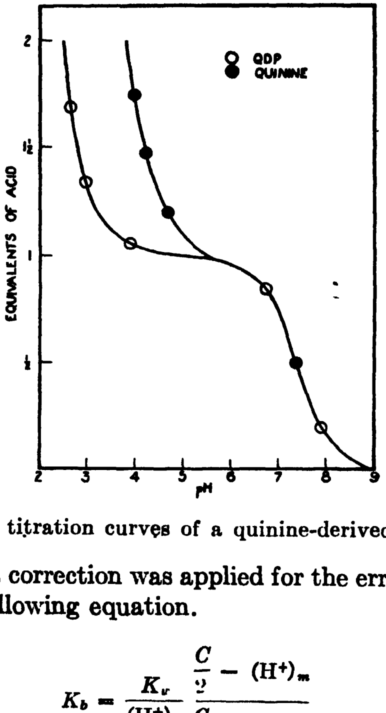

FIG. 1. Potentiometric titration curves of a quinine-derived product and quinine

of quinine or QDP, a correction was applied for the error due to hydrolysis by the use of the following equation.

$$K_b = \frac{K_w}{(H^+)_m} \frac{\frac{C}{2} - (H^+)_m}{\frac{C}{2} + (H^+)_m}$$

where K_b is the basic dissociation constant, C is the total concentration of the base at the half neutralization point, and $(H^+)_m$ is the observed hydrogen ion activity at that point. When the effect of ethanol is disregarded, the calculated dissociation constants at 25° are as follows: quinine, $K_{b_1} = 2.8 \times 10^{-7}$ (82 per cent ethanol), $K_{b_2} = 1.8 \times 10^{-10}$ (70 per cent ethanol); QDP, $K_{b_1} = 2.8 \times 10^{-7}$ (82 per cent ethanol), $K_{b_2} = 2.3 \times 10^{-12}$ (70 per cent ethanol). The titration curves are given in Fig. 1.

Absorption Spectra—The absorption curves of quinine, QDP, and 2-hydroxy-6-methoxy-4-methylquinoline[1] (6-methoxy-4-methylcarbostyril) were investigated with a Beckman quartz spectrophotometer in 0.0002 M ethanol solutions from 250 to 380 mμ. The hydrogen lamp was used to 320 mμ and a filter added to 380 mμ. The latter values were checked with a tungsten lamp.

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[1] Prepared by Mr. Alf Reims; compare Ainley and King (3).

With a cell thickness of 1 cm. and at a temperature of about 26°, quinine showed maxima at 279 and 333 mμ and minima at 225 and 301 mμ. Under the same experimental conditions, QDP showed a maximum at 352 mμ and a minimum at 297 mμ, compared with a maximum of 350 mμ and a minimum of 294 mμ for 2-hydroxy-6-methoxy-4-methylquinoline. For purposes of comparison the three curves are plotted in Fig. 2.

Hydrogenation of QDP—At 22° and 741 mm., an ethanol solution containing Adams' catalyst and 9.021 mg. of QDP absorbed 0.667 ml. of hydrogen.[2] Assuming one olefinic linkage to have been hydrogenated, the molecular weight of QDP is calculated from this to be 336.

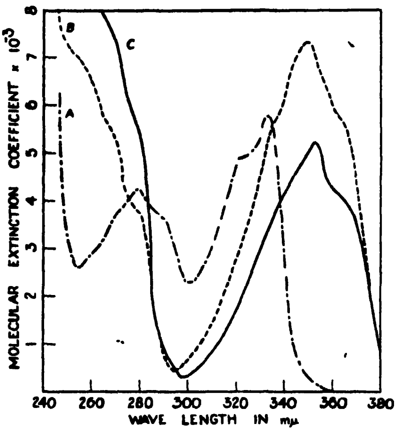

FIG. 2. Absorption spectra of quinine (Curve A), a quinine-derived product (Curve B), and 2-hydroxy-6-methoxy-4-methylquinoline (Curve C).

Ozonization of QDP—A suspension of 0.2828 gm. of QDP in 20 ml. of purified chloroform at 0° was treated with a stream of 5 to 6 per cent ozone at 100 ml. per minute for 4 hours (4). The chloroform was evaporated at room temperature and the colorless residue was warmed with 25 ml. of water on the water bath for 1 hour. 20 ml. of this solution were distilled into 5 ml. of ice water and then treated with 0.28 gm. of medon. When the mixture was warmed for 5 minutes on the water bath, a precipitate appeared which, after filtration and recrystallization from ethanol, melted at 188–190° (corrected) and gave a mixed melting point of 188–190°

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[2] The authors are indebted to Dr. G. Oppenheimer for carrying out this determination.

(corrected) with a known sample of formaldehyde dimedon. The recrystallized product weighed 0.0384 gm. or 16 per cent of the theory. Quinine, under the same conditions, gave a corresponding amount of formaldehyde dimedon.

Reaction with Methyl Iodide—An excess of methyl iodide was added to a solution consisting of 0.3 gm. of QDP which had been dissolved in 5 ml. of chloroform with the aid of a little methanol. After the solution was warmed, a heavy precipitate appeared which was filtered off, washed with chloroform, and dried to yield 0.42 gm. of crystalline solid. This was recrystallized from ethanol to give colorless needles, m.p. 276–277° (with decomposition; corrected).

$C_{21}H_{27}O_3N_2I$.	Calculated.	C 52.28,	H 5.64,	N 5.81,	I 26.31,	MeO 6.4
482.4	Found.	" 52.21,	" 5.63,	" 5.52,	"26.37,	" 6.7

This would indicate that QDP forms a monomethiodide which dissolves in dilute mineral acids and in bases, including NH_4OH.

When the reaction of QDP with methyl iodide was carried out in a sealed tube with methanolic KOH, a product could not be isolated. If a sample of the methiodide was refluxed in methanol with methyl iodide, the solution became acidic and the solid went into solution. A definite compound could not be isolated from this reaction mixture.

Reaction with Benzenesulfonyl Chloride—0.3 gm. of QDP was suspended in 3 ml. of 6 N KOH and warmed on the water bath; an oil formed which dissolved on dilution with 10 ml. of water. To this solution there was added 0.3 ml. of benzenesulfonyl chloride, the mixture was shaken for 1 hour, and an additional 0.1 ml. of benzenesulfonyl chloride was added. After another hour of shaking and stirring the mixture was warmed to decompose excess acid chloride. An oily precipitate, which had gradually formed and then solidified, was filtered off, washed with water, and dried. After one crystallization from ethanol and two from water the product melted at 179–180°, but still contained ash and gave a flame test for potassium. It was therefore recrystallized from water with the addition of a drop of HCl just at the point of crystallization. After three more crystallizations from water, long sheaves of colorless needles were obtained; m.p. 180–181° (corrected). A sample for analysis was unchanged after heating for 15 hours at 140° *in vacuo*.

$C_{58}H_{68}O_{16}N_4S_3$.	Calculated.	C 59.36,	H 5.84,	N 4.78,	S 8.20,	MeO 5.29
1173.3	Found.	" 59.44,	" 5.83,	" 4.78,	"8.22,	" 5.35

This compound is soluble in ethanol and hot water and insoluble in hydrocarbon solvents. The possibility that it is a salt of benzenesulfonic acid is excluded by the finding that QDP can be recovered unchanged from a solution containing a corresponding amount of benzenesulfonic acid. It is insoluble in dilute bases and slowly soluble in dilute mineral acids; from the latter solution, after 2 hours refluxing, QDP was recovered by neutralization with NH_4OH.

Miscellaneous Reactions of QDP—When heated for 3 hours in a sealed tube with water at 150°, or with 2 N HCl at 150°, QDP dissolved but was recovered unchanged on cooling or on neutralization of the acid solution. Moreover it was recovered unchanged after refluxing 45 hours with dilute acetic acid (compare von Miller and Rohde (5)).

QDP did not react with *p*-nitrophenylhydrazine, 2,4-dinitrophenylhydrazine, or hydroxylamine and was recovered unchanged from its absolute ethanolic sodium ethylate solution through which methyl nitrite had been passed (compare (5)).

With nitrous acid in dilute HCl, QDP gave a precipitate which gave the diphenylamine test and the Liebermann phenol test for nitrosamines. With nitrous acid in acetic acid solution it gave a deep yellow color. With ceric nitrate reagent QDP gave a deep red color; this color is given by carbostyril (2-hydroxyquinoline) but not by quinine.

In chloroform-methanol solution QDP took up bromine rapidly, forming a yellow precipitate which melted at about 280°.

Unsuccessful attempts were made to obtain a recognizable fragment of the QDP molecule after oxidation with a number of agents and under a variety of conditions. Thus, no quininic acid could be obtained after oxidation with chromic acid, although parallel experiments with quinine led to the isolation of good yields of quininic acid (6). In the case of the oxidation of QDP with dilute HNO_3 a crystalline product was obtained which proved to be a nitrate. This compound on treatment with dilute NH_4OH gave a red crystalline material which did not melt below 300°. Preliminary analyses of this compound indicated that degradation of the QDP molecule had not been effected; therefore further attempts at oxidation with HNO_3 were abandoned.

DISCUSSION

The following discussion summarizes the experimental evidence and the reasons for assigning the structure (II) to QDP.

The homogeneity of QDP is evidenced by its constant rotation and melting point after successive crystallizations and by its behavior when chromatographed.

The analyses are in excellent agreement for an empirical formula containing 1 more oxygen atom than that of quinine. It was therefore assumed that no deep seated structural change had taken place in the quinine molecule.

Although a direct molecular weight determination of QDP proved unsatisfactory, there is no evidence to suggest that the molecular weight is other than 340.4, as calculated from the simple empirical formula $C_{20}H_{24}O_3N_2$. On the contrary, the titration curve for QDP (Fig. 1) is not consistent with a double molecule. Secondly, ozonolysis indicated the presence of an expected vinyl group and quantitative hydrogenation confirmed an aliphatic double bond; from the hydrogenation data the molecular weight of QDP is calculated to be 336, based upon the reduction of one double bond, which is in good agreement with 340.4 for $C_{20}H_{24}O_3N_2$.

Evidence that the quinuclidine ring of quinine is retained unchanged in QDP is shown by the following: (*a*) the vinyl group is intact, as evidenced by ozonolysis, hydrogenation, and the addition of bromine; (*b*) QDP forms by addition a monomethiodide; (*c*) QDP does not react with methyl nitrite and therefore does not contain the grouping $—C^9O—C^8H_2—$ which would be present if the quinuclidine ring had been opened and a quinicine-like structure were involved; (*d*) the first basic dissociation constant, K_{b_1}, is the same for quinine and QDP.

Evidence that it is the quinoline portion of the molecule in quinine which has been altered in the formation of QDP is shown by the following: (*a*) quininic acid could not be obtained by oxidation of QDP; (*b*) in contrast to quinine, QDP is soluble in solutions of the hydroxides of the alkali metals; (*c*) QDP does not form a dimethiodide, as does quinine; (*d*) the second basic dissociation constant, K_{b_2}, for QDP is very small.

Consideration may now be given to the reasons for assigning to QDP the structure (II) containing an oxygen in the 2′ position of the quinoline ring. Since QDP is derived from quinine (I), the 4′ and 6′ positions must be occupied, the latter by the methoxyl group which has been shown to be present. The possibility of an amine oxide structure may be ruled out since QDP forms only a monomethiodide. The addition of an oxygen to the 3′, 5′, 7′, or 8′ position in quinine (I) would be expected to produce a phenolic compound of a sufficiently acidic nature to react with diazomethane and to give a color reaction with ferric chloride; QDP, however, does not react with either reagent. There remains therefore only the 2′ position to be occupied by the oxygen atom which must be accounted for in QDP; a hydroxyl group in this position, and *only* in this position, would account for acidic properties weaker than those shown by an ordinary phenolic hydroxyl group, as well as for the marked decrease in basic properties of the quinoline nitrogen.

Thus the structure (II) accounts very well for the properties of QDP and is in harmony with the experimental evidence. Such a structure may be looked upon as a 4,6-disubstituted carbostyril, and the absorption spectra shown in Fig. 2 are in excellent agreement with such a view. The acidic properties of QDP are comparable to those of a substituted carbostyril, for, although carbostyril itself reacts with diazomethane to give an O-methyl ether, the acidity decreases with substitution, and 6-methoxy-4-methylcarbostyril does not react with diazomethane. Moreover carbostyrils are not smoothly oxidized under acid conditions (7), which accounts for the fact that attempts to obtain recognizable oxidation products of QDP were unsuccessful. The slight basicity of the carbostyril nitrogen in QDP explains the nitrosamine test as well as the fact that on prolonged treatment with methyl iodide a solution of QDP becomes acid, without doubt by liberation of HI, although the N-methyl ether was not isolated.

There remain two experimental observations for which a satisfactory explanation cannot be offered at this time, although neither observation necessarily conflicts with the structure (II) for QDP. The first of these is that QDP apparently does not isomerize to a quinicine-like structure as does quinine. This may be due to the altered quinoline ring in QDP, since any rearrangement involving the grouping $\text{—C}^9\text{HOH—}\underset{|}{\text{C}^8\text{H}}\text{—}\underset{|}{\text{N}}\text{—}$ might well be influenced by a change in the quinoline portion of the molecule from that of structure (I) to (II). The second is the formation of the compound with a melting point of 179–180° by the action of an excess of benzenesulfonyl chloride on QDP in alkali. The analytical results are in close agreement with the calculated values for the empirical formula $C_{58}H_{68}N_4O_{16}S_3$. This empirical formula could be accounted for on the assumption that the reactants have combined in any one of several proportions; *e.g.*, 2 moles of QDP plus 3 moles of benzenesulfonyl chloride plus 4 moles of water with loss of 3 moles of HCl, or 2 moles of QDP plus 3 moles of benzenesulfonic acid plus 1 mole of H_2O. However, it has not been possible to write a structure which properly accounts for any such combination. The significant fact is that the compound is not likely to involve an S—N linkage, since QDP was recovered quantitatively after mild acid hydrolysis.

SUMMARY

Evidence is presented that the crystalline metabolic product, m.p. 247.5–248.5°, derived from quinine, is levorotatory 2′-hydroxy-6′-methoxy-3-vinylruban-9-ol.

The authors are indebted to Dr. F. E. Kelsey, Dr. E. M. K. Geiling, Dr. Frances K. Oldham, and Dr. Earl H. Dearborn of the University of Chicago for the gift of a generous sample of their metabolic derivative of quinine and to Mr. William Saschek, College of Physicians and Surgeons, Columbia University, for the microanalyses reported here.

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MODIFICATION OF THE CHLORATE DIGESTION METHOD FOR MICRODETERMINATION OF IODINE IN BIOLOGICAL MATERIALS

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(Received for publication, April 3, 1944)

In a previous paper[1] a method for the microdetermination of iodine was described. This consisted of digesting the organic material in sulfuric acid and potassium chlorate. The method, although very rapid, had certain limitations. By modifying it practically all the disadvantages have been overcome. One of the limitations was that not more than 30 mg. of solid matter could be used in each sample. According to the new method as much as 80 mg. of solid matter can be used for analysis. The second disadvantage was the interference of iron and manganese, which limited the determination to products that did not contain these materials. The cause of this trouble has also been eliminated. The third limitation was the restricted period of titration which was caused by the liberation of iodine from potassium iodide owing to high acidity. This has been avoided by the addition of phosphate, which actually has a 2-fold purpose, as will be explained in the following paragraph.

In the modified method, the material is digested in a solution consisting of perchloric acid, sodium chlorate,[2] and disodium hydrogen phosphate. During the digestion iodine is oxidized to iodic acid. When the digestion is complete, the liquid is made alkaline by the addition of sodium hydroxide to precipitate iron, calcium, and copper in the form of phosphate salts which are removed by centrifugation. The clear liquid is then acidified with hydrochloric acid to a pH of 1.2, with thymol blue as an indicator. At this pH in the presence of phosphate, ferric salts do not oxidize potassium iodide and, therefore, even if iron is not removed from the solution by centrifugation it will not interfere with the iodide-iodate reaction. The indicator is then decolorized with a solution of chlorine in carbon tetrachloride, which also oxidizes the reducing impurities that might have been present in the added reagents. After the chlorine is removed by boiling, the solution is cooled, a crystal of phenol is added, and the solution allowed to stand for 5 minutes; such concentrations of manganese salts as are found in tissues will then be reduced by the phenol after this time. Iodine is then liberated by the addition of potassium iodide solution. Soon after, the pH of the solution is adjusted to about 6 by the addition of disodium hydrogen phosphate solution. This change in pH has two functions. First, at this pH no iodine will be liberated from the potassium iodide solution and the titration can be carried out as slowly as desired; secondly, an almost neutral solution will safeguard against the oxidation of potassium iodide by slow oxidizing agents that might be present in the solution as impurities.

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[1] Shahrokh, B. K., *J. Biol. Chem.*, **147**, 109 (1943).

[2] Potassium salts cannot be used because on their addition to perchloric acid-potassium perchlorate precipitates, which interferes with the determination.

This method takes longer than the original method. It will take about 3 hours to determine the iodine content of twelve samples, if they are digested together, which will give an average of about 15 minutes for each determination. If the centrifugation of the precipitate is not necessary, as is the case with most tissues, the time can be reduced to about 2½ hours for twelve samples. There is almost 100 per cent recovery of iodine by this method if the directions are thoroughly followed. The accuracy of the method, therefore, is limited by the accuracy of titration. 0.5 γ of iodine is the limit of accuracy for the writer.

Reagents—

1. Digestion mixture. Disodium hydrogen phosphate (anhydrous) 2.5 gm., sodium chlorate 30.0 gm., distilled water 135.0 ml., 60 per cent perchloric acid 90.0 ml. First sodium chlorate and disodium hydrogen phosphate are dissolved in distilled water. Then, while the mixture is shaken slowly, perchloric acid is added. If the liquid becomes warm, it is cooled under tap water. The mixture should be kept at room temperature. Higher temperatures cause speedy decomposition of the reagents.

2. 13 per cent sodium hydroxide solution.

3. Thymol blue indicator. 0.04 per cent thymol blue in 50 per cent alcohol.

4. Solution of chlorine in carbon tetrachloride. (*a*) If chlorine gas is available, chlorine solution can be obtained by slowly bubbling chlorine through carbon tetrachloride. (*b*) If chlorine gas is not available, chlorine can be obtained from sodium hypochlorite solution. 100 ml. of 5 per cent sodium hypochlorite (commercial products like Clorox are satisfactory) are placed in a 250 ml. separatory funnel, followed by 25 ml. of carbon tetrachloride. The solution is then slowly acidified with 6 N HCl. After each addition the separatory funnel is shaken. When the liquid becomes deep green, the solution is ready for extraction. There will be evolution of CO_2 gas during and after acidification. This gas should be allowed to escape. The mixture is shaken slowly to dissolve the chlorine in the carbon tetrachloride. The shaking is continued until the hypochlorite solution loses its green color. Carbon tetrachloride is then drained out. The green tetrachloride solution is washed only once with 25 ml. of distilled

water. If the solution remains turbid, it is centrifuged for about 30 minutes in a stoppered 50 ml. centrifuge tube. This solution should be kept in a glass-stoppered bottle and must be prepared fresh every 3 or 4 weeks.

5. 1 per cent potassium iodide solution.
6. 5 per cent disodium hydrogen phosphate solution.
7. 0.001 N sodium thiosulfate solution freshly prepared before titration.
8. 1 per cent starch indicator.

Method

1 ml. of sample is pipetted into micro-Kjeldahl digestion flasks. It is preferable to use the type of digestion flasks which can also be used for centrifuging. Then, 6 ml. of the digestion mixture are added to the sample, followed by a few small pieces of pumice stone. The addition of pumice stone is essential to prevent the vigorous bumping which may occur. The flasks are heated on a digestion rack until the liquids start to turn green, when the micro flames are lowered and the flasks are heated very gently. During this part of the digestion the liquids should simmer and not boil. Speedy heating may cause minor explosions. The heating is continued in this manner until no more chlorine gas is seen in the neck of the flasks. This may take from 30 minutes to 1 hour, depending on the quantity of organic material present. When chlorine gas disappears, the flames are raised slowly and the liquids are allowed to boil. Vigorous boiling should be avoided in all stages of digestion. When the contents of the flasks are reduced to about 1 ml. to 2 ml., the solutions become colorless and fumes start to evolve. Heating is continued for another 2 to 3 minutes, after which the flasks are allowed to cool. 4 ml. of 13 per cent NaOH are then added to each sample. The flasks are cooled to room temperature with tap water. 1 drop of thymol blue is then added and sufficient 13 per cent NaOH to turn the indicator blue. If at this stage a visible precipitate appears, the liquid should be centrifuged for 1 minute. The supernatant liquids are poured in 50 ml. Erlenmeyer flasks, the precipitates are washed twice, each time with 5 ml. of distilled water, and the washings added to the original liquid.[3] Sufficient 1 N hydrochloric acid is added to each flask to give a definite pink color. A few drops of a solution of chlorine in carbon tetrachloride are added to each flask to decolorize the liquids. The samples are then boiled until about 15 ml. of liquid are left in each flask, after which they are cooled in a stream of running water. When the temperature reaches about 40° (at higher temperatures phenol reduces iodic

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[3] The author prefers to omit the centrifuging unless copper is present. If the solution is not centrifuged, small quantities of precipitate will remain in the liquid. However, in none of the biological materials encountered has the amount of precipitate been large enough to interfere with the accuracy of the titration.

acid), a crystal of phenol is added to each flask and, after cooling for at least 5 minutes, 1 ml. of 1 per cent potassium iodide solution is added to each flask. At a pH of approximately 1.2 the iodide-iodate reaction goes on at a comparatively slow rate and about 30 seconds should be allowed for complete liberation of iodine. When the reaction is complete, 1 ml. of a 5 per cent solution of disodium hydrogen phosphate is added, and the iodine is titrated with a freshly prepared 0.001 N solution of sodium thiosulfate, 1 drop of 1 per cent starch being used as an indicator.

When a large number of samples are being analyzed, the time for determination can be reduced if, while one series is boiling in Erlenmeyer flasks, another series is being prepared in digestion flasks and if while the titrations are being made for the first batch the second series can be digested.

SUMMARY

The chlorate digestion method for the microdetermination of iodine is modified so that this method can be used for all the biological materials containing more than 0.01 per cent of iodine.

THE EFFECTS OF SPERMINE, SPERMIDINE, AND OTHER POLYAMINES ON THE GROWTH INHIBITION OF ESCHERICHIA COLI BY ATABRINE*

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We have previously shown that the growth of *Escherichia coli* can be inhibited by atabrine (1). This inhibition can be prevented by the addition of Witte's peptone, by protein digests, and by small concentrations of spermidine, spermine, pantothenic acid, and several other compounds. The present report comprises a detailed description of these phenomena and preliminary observations as to their mechanism.

Materials and Methods

Cultures—*Escherichia coli* Strain 26 from the Department of Bacteriology, Iowa State College; *Lactobacillus casei* from the American Meat Institute; *Pseudomonas pyocyanea* from the Department of Bacteriology, University of Chicago.

Media—The mineral salts-glucose medium was that of Kohn and Harris (2). The medium was adjusted to pH 7.8 before being boiled and clarified by filtration. The final pH after sterilization was between 7.15 and 7.3.

Media with 1 per cent concentrations of peptone, yeast extract, and casein hydrolysate were prepared in 0.6 per cent NaCl and 0.2 per cent glucose. They were adjusted to pH 7.4 prior to sterilization. After sterilization the pH varied from 7.1 to 7.3.

The medium of Landy and Dicken (3) was employed for the growth of *Lactobacillus casei.*

2 per cent Mead's amigen adjusted to pH 7.6 before sterilization was used for the growth of *Pseudomonas pyocyanea.* 250 ml. portions were distributed into 4 liter Erlenmeyer flasks to insure a large surface area. Cells

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* The work described here was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. This work was also supported in part by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

The authors are indebted to the Carbide and Carbon Chemical Corporation for their gift of the synthetic polyamines employed. They also wish to express their thanks to Mr. M. Lieberman for the isolation of several soil organisms and to Miss Virginia Blue for much of the routine work.

were collected, after 18 to 20 hours growth, by centrifugation in a Sharples supercentrifuge.

All basal media were heat-sterilized at 15 pounds for 20 minutes. Solutions of substances added to the basal media were adjusted to pH 7.4 before sterilization. The final volume of the medium was 10 ml. in all cases. Aqueous atabrine dihydrochloride (0.025 M) and quinine dihydrochloride solutions (0.05 M containing 1 ml. of 0.5 N NaOH per 20 ml.) were sterilized by filtration through Jena glass sterilizing filters. Irregularities resulted when atabrine solutions were sterilized by passage through Seitz filters, probably due to absorption of the drug on the filter pad. Solutions of heat-labile substances were sterilized by filtration; all others were sterilized by autoclaving. Incubation temperatures were 32° unless otherwise specified.

Warburg manometers and vessels were employed in respiration measurements. Aldehyde formation was measured by a micro modification of the method of Clift and Cook (4).

EXPERIMENTAL

Effect of pH on Atabrine Inhibition—It is evident from Table I that the bacteriostatic action of atabrine on *Escherichia coli* is a function of the pH of the medium. Little inhibition of bacterial growth is observed below pH 6.09, with atabrine concentrations as high as 0.0015 M. On the other hand, a measurable degree of inhibition is still apparent with atabrine concentrations of 0.00025 M at pH 7.57. Atabrine is apparently most effective as the free base.[1]

To ascertain whether the increased activity of atabrine at higher pH levels could be correlated with an increased concentration of the drug inside or on the surface of the bacterial cells, experiments were carried out in which washed suspensions of *Escherichia coli* in water were added to buffers of different pH values containing identical concentrations of atabrine. The suspensions were shaken for 5 minutes at room temperature, the cells were centrifuged off, and 1 ml. samples of the supernatant fluid were taken for analysis. The atabrine was extracted with 15 ml. of ethylene dichloride in the presence of 1 ml. of saturated Na_2HPO_4 (6). The intensity of the fluorescence of the ethylene dichloride extracts was compared on the Coleman electronic photofluorometer, with Coleman Filters B-2 and PC-2. An increase in the external pH of the medium from 6.2 to 7.6 was found to cause an 8-fold increase in the percentage of atabrine bound or contained in the cells (Table II).

Antagonistic Properties of Peptones, Yeast Extract, and Casein Hydrolysates—A variety of materials was tested for the content of substances capable of antagonizing the inhibitory action of atabrine. The pertinent data are included in Table III.

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[1] Browning, Gulbransen, and Kennaway (5) have already pointed out that acridine compounds were most effective against *Escherichia coli* in alkaline media.

Bacto-yeast extract and one sample of peptone (No. 358,027) contained amounts of antagonizing substances capable of removing the inhibiting effect of 0.0005 M atabrine in 11 and 12 hours respectively. Growth in the control tubes in these experiments, *i.e.* in the absence of atabrine, occurred in 5 hours. A second sample of Bacto-peptone (No. 348,562) contained smaller amounts of the antagonistic factors.[2] When amigen was present, growth occurred in the presence of 0.00125 M atabrine in about twice the time (11 hours) required for the control. With the same high concentration

TABLE I

Effect of pH on Growth Inhibition of Escherichia coli by Atabrine

Growth was initiated in 10 ml. of Bacto-peptone with 1 drop of the 24 hour growth of *Escherichia coli* in Bacto-peptone diluted 1:5 with distilled water.

pH	Atabrine concentration			
	0.00025 M	0.0005 M	0.001 M	0.0015 M
	Hrs. for visible growth (<7 for 0.0 M atabrine)			
5.70	<7	<7	<7	<7
6.09	<7	<7	<7	<7
6.23	<7	<7	<7	48
6.53	<7	<7	>96	>96
6.83	<7	10	>96	>96
7.21	<7	36	>96	>96
7.38	<7	48-60	>96	>96
7.57	9.5	48-60	>96	>96

TABLE II

Effect of pH on Distribution of Atabrine between Bacterial Cells and Suspending Medium

2 ml. of phosphate buffer (M/15); 1 ml. of atabrine (244.8 γ as free base); 1 ml. of 4 per cent *Escherichia coli* suspension in water; incubated with shaking for 5 minutes at room temperature and centrifuged.

pH	Atabrine concentrations		Ratio $\frac{\text{cells}}{\text{supernatant}}$
	Supernatant	Cells	
	γ per ml.	*γ per ml.*	
6.2	57.7	350	6.1
7.6	31.4	2980	95.0

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[2] Unless otherwise indicated, this sample of peptone was used in the work reported here.

of atabrine, the presence of Witte's peptone permitted growth after a lag of only 0.5 hour (*i.e.*, 5.5 hours). With concentrations of atabrine less than 0.0005 M, the presence of Witte's peptone completely prevented growth inhibition. Various casein hydrolysates were essentially without activity. Whether the activity of these various materials is due to any of the substances discussed in the following section is unknown.

TABLE III

Effect of Various Media on Atabrine Inhibition of Escherichia coli Growth

Inoculum, 1 drop of the 24 hour *Escherichia coli* growth in Bacto-peptone diluted 1:1000 with H_2O.

pH	Medium	Atabrine concentration						
		None	0.000125 M	0.00025 M	0.0005 M	0.00075 M	0.001 M	0.00125 M
		Hrs. for visible growth						
7.16	Bacto-yeast extract	5		6 5	12	>48		
7.28	Bacto-peptone, No 348,562	5		7.5	>48			
7.31	Witte's peptone	5		5	5	5.5	5.5	5 5
7.28	Bacto-peptone, No. 358,027	5		6.5	11	>48		
7.27	Bacto-casamino acids	6 5		>48				
7.15	Mead's amigen	5		6	6.5	7.5	9	11
7.15	Mineral salts-glucose	15	20	24	>48			

Pollack and Lindner (7) have demonstrated the presence of a substance in Wilson's peptone which serves as a growth stimulant for *Lactobacillus casei*. In order to ascertain whether Witte's peptone and the other preparations tested on *Escherichia coli* were effective as growth stimulants for *L. casei* we have carried out experiments in which these substances were added to the medium of Landy and Dicken (3) at a concentration of 20 γ per ml. The media were then inoculated with 1 drop of a washed *L. casei* suspension and incubated at 37° and the turbidity, expressed in arbitrary units as determined on the Evelyn photometer, caused by the resultant growth measured after 24 hours. The results were as follows:

Media added	Amigen	Bacto-peptone, No. 348,562	Bacto-peptone, No. 358,027	Bacto-yeast extract	Bacto-technical casamino acids	Witte's peptone	Control
Growth	13.7	13.7	11.3	9.1	7.1	29.2	9.7

Witte's peptone contained a factor, lacking or present in only small quantities in the other media, which markedly increased the growth of *L. casei*. Very little of this factor was present in amigen. Whether the factor or factors in Witte's peptone which stimulated the growth of *L. casei* are identical with those which antagonized the effect of atabrine on the growth of *E. coli* is not known.

Chemically Known Factors and Their Effect on Atabrine Antagonism—Data showing the effect of a number of chemically pure substances on the growth of *Escherichia coli* in the presence of atabrine are presented in Table IV.

These tests were carried out in a medium containing 1 per cent Bacto-peptone. With this medium the bacteriostatic effect of atabrine is only slightly less than in an inorganic salts-glucose medium and no increase in the control growth rate (in the absence of atabrine) was caused by the addition of the substances being tested. Conditions were so adjusted that growth in the controls (in the absence of atabrine) occurred in about 5 hours. In the presence of 0.0005 M atabrine, visible growth did not occur before 24 hours and frequently not until 48 hours. Only those compounds were considered active which, in the presence of atabrine, caused visible growth within 24 hours.

The naturally occurring polyamines, spermine and spermidine, were the most active of the substances tested. Pantothenic acid and the synthetic amines, diethylenetriamine, etc., were less active. Cystine, riboflavin, thiamine, nicotinic acid, and glutathione showed some activity, while the effects of tryptophane, 1,4-diaminobutane, and *l*-arginine were irregular and small. With the exception of riboflavin, none of the active substances shows any chemical resemblance to atabrine, and there is no obvious structural similarity between the various groups of active compounds.

Relative Toxicities and Antagonistic Properties of Spermine and Spermidine—The antagonistic effect of low concentrations of spermine to atabrine is replaced, with higher concentrations of the amine, by a direct inhibitory effect on the growth of *Escherichia coli*. On the other hand, spermidine, which is also highly effective in antagonizing atabrine, does not inhibit the growth of *Escherichia coli* at 0.002 M concentrations (Table V). At lower concentrations, however, spermine is the more effective antagonist of the two amines. As shown in Table VI, 1.2 γ per ml. (6.25×10^{-6} M) of spermine are capable of effectively antagonizing 327 γ per ml. (7.5×10^{-4} M) of atabrine.

Effect of Spermidine on Growth of Organisms Other Than Escherichia coli in Presence of Atabrine—To ascertain whether the spermidine-atabrine antagonism demonstrated with *Escherichia coli* could be observed with other bacteria, organisms isolated from soil were studied under conditions similar to those used with the colon organism.

It was found that these soil organisms can be divided into two groups. The first group comprises those which are inhibited by low concentrations

Table IV

Factors Antagonizing Inhibiting Effect of Atabrine on Growth of Escherichia coli

Growth was initiated in the basal medium of Bacto-peptone with 1 drop of the 24 hour growth of *Escherichia coli* in Bacto-peptone diluted 1:1000.

The atabrine concentration in all experiments was 0.0005 M.

Factor	Concentration	Hrs. required for initiation of visible growth
	M	
Spermidine	0.001	6
	0.0005	6
	0.00025	7
Spermine	0.001	22
	0.0005	10
	0.00025	7
Diethylenetriamine	0.001	10
	0.0005	20
	0.00025	24
Triethylenetetramine	0.001	7
	0.0005	9
	0.00025	23
Tetraethylenepentamine	0 001	6.5
	0.0005	7.5
	0.00025	20
Calcium pantothenate	0.001	6.5
	0.0005	9
	0.00025	20
Riboflavin	0.00033	16
Thiamine	0.00037	14–16
Nicotinic acid	0.0010	14–16
Glutathione	0.00041	14–16
Cystine	0.0005	18

The following show slight and variable activity at the concentrations indicated: tryptophane 0.0006 M, putrescine 0.0008 M, *d*-arginine 0.0006 M. The substances inactive at concentrations of 125 γ per ml. were as follows: ethylamine, butylamine, monoamylamine, dimethylamine, diethylamine, triamylamine, isopropylamine, ethylenediamine, propylenediamine, monoethanolamine, triethanolamine, phenyldiethanolamine, triisopropanolamine, phenylethanolamine, ethylphenylethanolamine, tetraethanolammonium hydroxide, diethanolamine, diethanolaminoethanol, morpholine, tri-*n*-butylamine, di-*n*-butylamine, histamine, *dl*-α-alanine, β-alanine, aminoacetic acid, *l*-asparagine, *dl*-aspartic acid, *l*(+)-arginine, *l*(+)-glutamic acid, *dl*-glutamic acid, histidine, *dl*-leucine, *dl*-isoleucine, *l*(−)-leucine, *l*(+)-lysine, *dl*-lysine, *dl*-norleucine, *dl*-methionine, *d*-ornithine, *dl*-phenylalanine, *l*(−)-proline, *l*(−)-hydroxyproline, *dl*-serine, *l*(−)-tyrosine, *dl*-threonine, *dl*-valine, aminobutyric acid, glutamine, adenine sulfate, choline, creatine, inositol, 2-methyl-1,4-naphthoquinone (62.5 γ per ml), *p*-aminobenzoic acid, pimelic acid, pyridoxine, uracil, uric acid, xanthine.

of atabrine (about 0.00005 M) when grown in peptone. These organisms are incapable of growth in an inorganic salts-glucose medium. The atabrine bacteriostasis with these organisms cannot be relieved by spermidine or Witte's peptone. The second group of organisms requires high (0.0005 M or more) concentrations of atabrine for inhibition of growth in peptone and is capable of cultivation in inorganic salts-glucose media. Atabrine bacteriostasis with these organisms can, as in the case of *Escherichia coli*, be relieved by spermidine. The data are shown in Table VII. It appears then that those bacteria which respond to spermidine can synthesize cellular protoplasm from a medium in which ammonium phosphate is the sole source of nitrogen. Those which require more complex media for growth (peptones) do not respond to the addition of spermidine. This suggests that at least two mechanisms are involved in atabrine bacteriostasis, one involved with the synthesis and metabolism of amines and another, yet unknown.

TABLE V

Relative Toxicity of Spermine and Spermidine

The inoculum and basal medium were as in Table IV.

	Concentration				
	0.0 M	0.00025 M	0.0005 M	0.001 M	0.002 M
	Hrs. for visible growth				
Spermine. . . .	4.5	5	9	20	
Spermidine.....	4.5	4.5	4.5	4.5	4.5

TABLE VI

Effect of Spermine and Spermidine on Growth of Escherichia coli in 0.00075 M Atabrine

The basal medium was Bacto-peptone No. 358,027. The inoculum was as in Table IV. Growth in the absence of atabrine occurred in 4.5 hours; with atabrine no growth occurred in 120 hours.

	7.5×10^{-4} M	5×10^{-4} M	2.5×10^{-4} M	1.25×10^{-4} M	6.25×10^{-5} M	2.5×10^{-5} M	1.25×10^{-5} M	6.25×10^{-6} M	2.5×10^{-6} M
	Hrs. for visible growth								
Spermine ..		6.75	5.75	5.5	5.5	8.5	20	26	>120
Spermidine .	5.75	5.75	8	14	26	>120			

Spermidine As Growth Factor?—The possibility that spermidine is an essential bacterial growth factor was tested on two organisms, with negative results. In the case of *Escherichia coli*, growth of the organism through a series of 120 daily transfers in either Bacto-peptone containing spermidine and atabrine or in an inorganic salts-glucose medium containing spermidine did not produce, at any time, organisms capable of increased growth in a mineral salts medium supplemented by spermidine. Attempts to isolate spermine and spermidine from 50 gm. (wet weight) of *Escherichia coli* cells by the usual methods (8) were unsuccessful.

The addition of spermidine was also found to be without effect on *Lactobacillus casei* growing on the medium of Landy and Dicken. When the casein hydrolysate used in the basal medium was adjusted to pH 10.8, extracted continuously with butyl alcohol for 40 hours, and then neutralized and used as the basal medium, it no longer supported growth. While the addition of spermidine to the extracted hydrolysate did not restore the growth-promoting property of the medium, normal growth was resumed when the butyl alcohol extract was added.

TABLE VII

Effect of Spermidine on Atabrine Inhibition of Growth of Soil Bacteria

The inoculum and basal medium were as for *Escherichia coli* in Table IV.

Organism No.	Addition	Growth at	
		17 hrs.	24 hrs.
1A	None	+	4+
	0.001 M atabrine	−	3+
	0.001 " " + 0.0005 M spermidine	+	4+
1	None	2+	4+
	0.0005 M atabrine	−	−
	0.0005 " " + 0.0005 M spermidine	+	4+
2	None	3+	4+
	0.0005 M atabrine	−	2+
	0.0005 " " + 0.0005 M spermidine	3+	4+
3	None	3+	4+
	0.00075 M atabrine	−	+
	0.00075 " " + 0.0005 M spermidine	2+	4+
5	None	2+	4+
	0.0005 M atabrine	−	2+
	0.0005 " " + 0.0005 M spermidine	2+	4+

Organism 1A, Gram stain irregular, short plump rod; dry wrinkled growth on agar. No. 1, Gram-negative, short slim rod; yellow smooth glistening growth on agar. No. 2, Gram-negative, resembling *Escherichia coli* morphologically and in growth on agar. No. 3, Gram-negative, short rod; yellowish smooth growth on agar. No. 5, Gram-negative, short plump rods; yellow growth on agar.

− = no growth; 4+ = maximum growth; +, 2+, 3+ = intermediate growth intensity estimated visually.

Quinine Bacteriostases and Spermidine—The growth of *Escherichia coli* on an inorganic salts-glucose medium can be inhibited by quinine although higher concentrations are required than with atabrine. The inhibition can be relieved by spermidine. This is shown when the effects of the addition of atabrine and quinine on growth of *Escherichia coli* in an inorganic salts-glucose medium are compared. With 1 drop of the 24 hour growth in mineral salts of pH 7.05 (2) diluted 1:1000 as the inoculum, the hours for visible growth after the addition were as follows: no addition, 18; 0.0005 M spermidine, 15; 0.0005 M atabrine, >72; 0.0005 M atabrine + 0.0005 M spermidine, 23; 0.0015 M quinine, 50; 0.0015 M quinine + 0.0005 M spermidine, 29. Whether the acceleration of growth evident on the addition of 0.0005 M spermidine to an inorganic salts-glucose medium is due to the amine serving as a source of organic nitrogen for cell synthesis or to some other effect is unknown. With a peptone medium, as stated previously, spermidine has no apparent effect on growth rate.

Attempts to demonstrate a chemical alteration of atabrine during bacterial growth in the presence of spermine and spermidine have been negative. In experiments to this end, the atabrine was extracted as the free base with ethylene dichloride after the medium had been made alkaline with Na_2HPO_4. After suitable dilution of the extracts with ethyl alcohol, the respective intensities of their colors were compared in a Coleman universal spectrophotometer with Filter PC-4 at the 430 mμ band. No difference in atabrine concentration could be detected between the tubes in which growth had occurred (in the presence of spermidine) and those in which no growth had taken place. This does not exclude the possible formation of metabolic products of atabrine having the same fluorescence and intensity of absorption at 430 mμ as atabrine itself, such as those described by Scudi and Jelinek (9). However, if such compounds are formed they must possess the same bacteriostatic potency as atabrine, inasmuch as the pigment extracted after bacterial growth in the presence of atabrine and spermine retains the full bacteriostatic effect of the atabrine originally added.

Development of Cultures Resistant to Atabrine—It was possible to obtain an *Escherichia coli* culture capable of growth in 0.0075 M atabrine by daily transfers of the organism to a mineral salts-glucose medium containing increasing concentrations of atabrine. After five transfers at the 0.0075 M atabrine concentration the organisms grew more slowly. The organism was then transferred to an agar slant and this was used as a source of the inoculum of the "atabrine-resistant" strain. Microscopic examination of the "resistant" organisms revealed a Gram-negative coccoid rod unlike the typical Gram-negative short slim rod of the parent culture. The identity of the culture was demonstrated by routine tests for *Escherichia coli:* (1) acid and gas production in lactose broth, (2) methyl red positive, (3) Voges-Proskauer negative, (4) no growth in Koser's citrate, (5) typical colonies on Endo's agar and Levine's eosin-methylene blue agar, and (6) indole production from tryptophane broth.

Table VIII shows the difference in resistance to both atabrine and quinine between normal and "atabrine-resistant" strains of *Escherichia coli*. Increased resistance to atabrine is accompanied by an increase in resistance toward quinine, although the latter (about 4 times) is not as great as the increase in atabrine resistance (about 10 times).

Inhibition of Spermidine Oxidation by Atabrine and Quinine—The fact that spermidine, spermine, histamine, and other polyamines are not oxidized by washed suspensions of the strain of *Escherichia coli* used in our experiments does not exclude the possibility that other metabolic transformations of the amines occur in *E. coli* since (1) the growth of *E. coli* in a glucose-inorganic salts medium is improved by the addition of spermidine and (2) (as reported by Dr. L. Peters and Dr. A. K. Miller in a personal communication) the growth of *E. coli* on a basal medium containing asparagine is accelerated by the addition of spermidine. It seemed desirable, therefore, to study the effect of atabrine and quinine on an enzyme system capable of using spermidine and other polyamines as a substrate. For this purpose we have used preparations of *Pseudomonas pyocyanea*, an organism capable of oxidizing putrescine, cadaverine, histamine, and other polyamines (10).

TABLE VIII

Resistance to Atabrine and Quinine of Normal and Atabrine-Resistant Strains of Escherichia coli

Basal medium, mineral salts-glucose. Inoculum, 24 hour cultures grown in mineral salts; 1 drop of culture diluted 1:1000. The data are expressed as in Table VII.

	Concentration of atabrine or quinine	Resistant strain			Normal strain		
		24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
	M						
Atabrine	None	+	4+	4+	4+	4+	4+
	0.0005				–	–	–
	0.001	–	4+	4+	–	–	–
	0.0015	–	4+	4+			
	0.002	–	4+	4+			
	0.003	–	4+	4+			
	0.004	–	4+	4+			
	0.005	–	–	4+			
Quinine	None	+	4+	4+	4+	4+	4+
	0.001				3+	4+	4+
	0.002	–	4+	4+	–	–	–
	0.003	–	4+	4+	–	–	–
	0.004	–	+	4+	–	–	–
	0.006	–	–	–	–	–	–

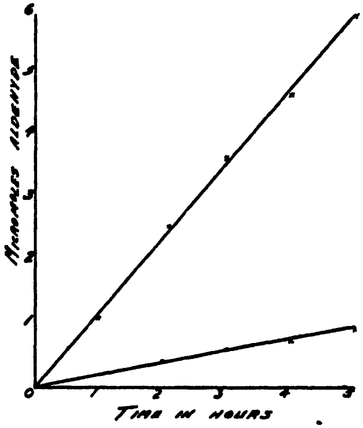

FIG. 1. The formation of aldehyde from spermidine by a lyophilized preparation of *Pseudomonas pyocyanea*. Lower curve, endogenous control; upper curve, in presence of spermidine. Volume, 2.5 ml. containing 1 ml. of M/15 PO_4, pH 7.6, 12.5 × 10^{-6} mole of spermidine, 28 mg. of undialyzed preparation, and H_2O to volume. Incubated at 30°.

TABLE IX

Effect of Atabrine and Quinine on Oxidation of Spermidine

0.5 ml. of phosphate buffer, pH 7.6, + 2.0 ml. (14 mg.) of lyophilized preparation of *Pseudomonas pyocyanea* (dialyzed 16 hours at 0°) incubated with shaking at 30°.

	Moles × 10^{-6} aldehyde formed in 1 hr.					
Spermidine	0.001 M			0.004 M		
Atabrine	None	0.0005 M	0.001 M	None	0.0005 M	0.001 M
Sample 1	1.57	0.94	0.75	1.79	1.60	1.27
Inhibition, %		40	52		10	29
Sample 2	1.17	0.71	0.47	1.40	1.29	0.96
Inhibition, %		40	60		8	32
Quinine	None	0.001 M	0.002 M	None	0.001 M	0.002 M
Sample 1	1.67	1.36	0.99	1.97	2.02	1.74
Inhibition, %		18	41			12
Sample 2	1.60	1.15	0.80	1.90	1.90	1.50
Inhibition, %		28	50		0	21
Sample 3	1.15	0.75	0.45	1.40	1.15	0.87
Inhibition, %		35	61		18	38

Suspensions of *Pseudomonas pyocyanea* oxidized spermine, spermidine, and putrescine at a rapid rate, histamine more slowly, and the three synthetic amines, diethylenetriamine, triethylenetetramine, and tetraethylenepentamine, at a very slow rate. If the cell suspensions were stored at 10° for 16 to 20 hours, it was found that atabrine markedly inhibited the oxidation of spermidine when it was added prior to the amines; if both amines are added simultaneously, or if freshly prepared suspensions of *Pseudomonas pyocyanea* were used, the inhibition was much less.

Such results, obtained with intact bacterial cells, are open to the objection that one cannot differentiate between the effects of inhibitors on the initial oxidation of the amine to the corresponding aldehyde and the subsequent oxidation of the latter. It was found, however, that lyophilized preparations of *Pseudomonas pyocyanea* formed an aldehyde (the primary product of diamine oxidase oxidation, according to Zeller (11)) with spermidine as a substrate (Fig. 1). Such preparations, after dialysis against distilled water, utilize from 1 to 2 atoms of O_2 per mole of aldehyde formed. This type of preparation has been employed for a study of the effect of atabrine and quinine on spermidine oxidation.

As is evident from Table IX, both quinine and atabrine inhibit the formation of aldehyde from spermidine. Furthermore, in each case, an increase of spermidine from 0.001 to 0.004 M greatly reduces the degree of inhibition, suggesting that the inhibition is competitive in nature.

DISCUSSION

The most striking fact to emerge from this study is the antagonism to the action of atabrine shown by the amines, spermine and spermidine. Since atabrine is apparently completely recovered after bacterial growth, the amine cannot be effective by combining with atabrine (unless the process is reversible) or by inducing the bacterial production of a substance capable of combining with or inactivating atabrine. It seems very probable that atabrine and spermine or spermidine are competitive substrates in an enzymic reaction occurring in the bacterial cell. Such a hypothesis implies that the bacterial synthesis of the amines or some other reaction involving them is necessary for the growth of the organism. Under circumstances in which any of these reactions is inhibited by atabrine, addition of the amine is necessary for growth to occur. Failure to isolate spermine or spermidine from *Escherichia coli* may be due to the fact that the amine is present in very small amounts. If one were to assume that the maximum concentration of the amine in the bacterial cell was equivalent to that necessary to overcome atabrine bacteriostasis, it can be calculated that the quantity present in 50 gm. of *Escherichia coli* would be too small to detect by our present techniques.

It seems unlikely that the ability of such agents as thiamine, riboflavin, pantothenic acid, glutathione, and nicotinic acid to antagonize atabrine is related to their various specific metabolic effects. The concentrations of these substances required to antagonize atabrine are far greater than those involved in their action on other biological systems. Further, any specificity in action is contraindicated by the fact that any one of the group in sufficiently high concentration will permit growth to occur. It seems more probable that all of these substances have some affinity for the active enzyme surfaces involved in the atabrine effect and protect these surfaces from the drug but do not completely inhibit the synthesis or other reactions of the amines essential for growth. Some support for this view may be derived from the work of Zeller (11) who has found that thiamine is an inhibitor of the diamine oxidases and has a great affinity for the enzyme.

The activity of the three synthetic polyamines increases with molecular weight and the number of amino groups (from three to five). At higher concentrations tetraethylenepentamine is almost equivalent in activity to spermidine. However, at a concentration of 0.00025 M the naturally occurring triamine is far more potent than the synthetic pentamine.

Those factors known to be the most active antagonists of the sulfonamides (*p*-aminobenzoic acid and methionine) are without effect on the action of atabrine. Witte's peptone, which contains the most active antagonists of the atabrine effect on *Escherichia coli*, has been reported by MacLeod (12) to be a very poor antagonist of the action of sulfonamides. It appears, therefore, that the mechanism of the action of atabrine is different from that of the sulfonamides. A similar conclusion has been reached by Maier and Riley (13) who found that *p*-aminobenzoic acid completely inhibited the antimalarial action of sulfanilamide in avian malaria, but was without effect on the action of quinine and atabrine.

Witte's peptone apparently contains other atabrine antagonists than the known polyamines, since it would be necessary for the peptone to contain about 0.1 per cent of the amines to account for its total activity. Further, Witte's peptone completely eliminates any lag in growth in 0.0005 M atabrine in contrast to the 1 hour lag always observed when optimum concentrations of the active amines are added.

SUMMARY

Increase in pH markedly increases the bacteriostatic activity of atabrine. This is associated with a greater retention of the drug by the cell at the higher pH values.

A variety of protein digests contains substances relieving atabrine bacteriostasis in *Escherichia coli*. Witte's peptone is the most active of these media. This same peptone in a concentration of 20 γ per ml. greatly increases the growth of *Lactobacillus casei* in the complete medium of Landy and Dicken.

The naturally occurring amines, spermine and spermidine, are the most

active antagonists of atabrine among the known factors tested. Concentrations of spermine higher than 0.00025 M inhibit the growth of *Escherichia coli;* spermidine is non-toxic at a concentration of 0.002 M.

Escherichia coli cultures resistant to atabrine also possess increased resistance to quinine. The bacteriostatic effect of quinine may be antagonized by spermidine.

The oxidation of spermidine by lyophilized preparations of *Pseudomonas pyocyanea* is inhibited by both atabrine and quinine. This inhibition may be eliminated or reduced by increased spermidine concentrations.

It is suggested that atabrine inhibits the growth of *Escherichia coli* by interfering with reactions involved in the synthesis, the metabolism, or the synthesis and metabolism of spermine or spermidine.

Addendum—Subsequent to the completion of this manuscript Snell (14) has reported that polyamines are effective in inhibiting bacteriostasis due to propamidine.

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RÔLE OF PHOSPHATE IN PYRUVIC ACID DISSIMILATION BY CELL-FREE EXTRACTS OF CLOSTRIDIUM BUTYLICUM*

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(Received for publication, April 8, 1944)

The preparation of a vacuum-dried cell-free extract of frozen cells of *Clostridium butylicum* has been described (1). This extract catalyzes the fermentation of pyruvic acid to acetic acid, carbon dioxide, and molecular hydrogen. The rate of hydrogen evolution during the reaction is proportional to the concentration of added inorganic phosphate. No stable phosphorylation product has been detected.

In this paper the results of a further study of the rôle of phosphate in the reaction are given.

EXPERIMENTAL

Large Scale Preparation of Cell-Free Extract—To avoid differences in the behavior of small batches of enzyme preparation individually prepared as needed, a large quantity of dry extract was prepared. Cells of *Clostridium butylicum* were grown as follows: 600 liters of medium consisting of 1 per cent commercial glucose, clear aqueous extract of 0.5 per cent malt sprouts and 0.25 per cent fresh pork liver, 0.25 per cent Cuban blackstrap molasses, 0.25 per cent ammonium sulfate, 0.1 per cent phosphoric acid neutralized to pH 6.8 with ammonium hydroxide, and salts,[1] was sterilized, cooled, and inoculated with 30 liters of an 18 hour culture of *Clostridium butylicum* grown in the same medium. After incubation at 37–40° for 18 hours, the cells were harvested by centrifuging. 900 gm. of wet cells were obtained and were immediately frozen.

After 14 days, 100 gm. portions of frozen cells were ground in 200 ml. of ice-cold water and adjusted to pH 6.5 with sodium hydroxide solution. The suspension was allowed to stand in an ice bath for 10 to 15 minutes and then centrifuged several times for 10 to 15 minute periods until a clear amber supernatant liquid could be drained from the cell débris. This liquid was evaporated under a high vacuum while frozen. From 700 gm. of frozen cells thus treated, 28 gm. of dry extract were obtained. Assay indicated recovery of 36 per cent of the total activity of the frozen cells used.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Wisconsin Alumni Research Foundation.

[1] A solution having the following content (in gm. per liter) was prepared: $MgSO_4 \cdot 7H_2O$ 10, $MnSO_4 \cdot H_2O$ 10, $CaCl_2 \cdot 2H_2O$ 10, $FeSO_4$ 0.2, $CuSO_4 \cdot 5H_2O$ 0.1, $Co(NO_3)_2 \cdot 6H_2O$ 0.01, $Na_2B_4O_7 \cdot 10H_2O$ 0.01, $ZnSO_4$ 0.1. 10 ml. of this solution were added per liter of medium prepared.

Determination of Inorganic Phosphate and Phosphate Esters—For convenience, the nomenclature given in Table I will be used in the discussion of phosphate compounds. These compounds were determined as follows: *True inorganic and labile phosphates* were determined concomitantly by a procedure modified from that of Lipmann (2). This procedure depends on the solubility of the calcium salts of labile phosphates and the insolubility of the calcium salts of true inorganic phosphate in alcoholic calcium chloride solution under controlled conditions, and on the hydrolysis of labile phosphate, if present, to inorganic phosphate during the determination of inorganic phosphate by a modification of the method of Fiske and Subbarow (3).

TABLE I

Nomenclature of Phosphate Compounds

Compound	Definition
Inorganic phosphate .	Phosphate whose calcium salt is insoluble in water-alcohol mixtures under controlled conditions (*e.g.*, NaH_2PO_4)
Labile phosphate.....	Phosphate whose calcium salt is soluble in water-alcohol mixtures under controlled conditions, and which is hydrolyzed, if present, during determination of inorganic phosphate by modified Fiske and Subbarow method (*e.g.*, acetyl phosphate)
7 minute phosphate.... .	Phosphate not hydrolyzed during Fiske and Subbarow determination of inorganic phosphate, but hydrolyzed by exposure to 1.0 N HCl at 100° for 7 minutes (*e.g.*, adenosine triphosphate)
Stable ester phosphate....	Phosphate not appreciably hydrolyzed by exposure to 1.0 N HCl at 100° for 7 minutes (*e.g.*, hexose diphosphate)

In the modified Fiske and Subbarow method referred to, 1 ml. of acid-molybdate reagent (5 gm. of ammonium molybdate plus 100 ml. of 10 N sulfuric acid diluted to 200 ml. with water) and 1 ml. of reducing reagent (14.25 gm. of sodium bisulfite, 0.25 gm. of 1-amino-2-naphthol-4-sulfonic acid, and 10 ml. of 5 per cent sodium sulfite solution made up in 80 ml. of water, dissolved, and diluted to 250 ml.) are added to 10 ml. of sample solution containing between 0.1 and 1.0 micromole of inorganic phosphate. After 10 minutes at room temperature, the blue color developed is compared in the Evelyn colorimeter at a wave-length of 660 mμ with the color produced by standard samples of inorganic phosphate. Labile phosphate, if present in the sample, is completely hydrolyzed and appears as inorganic phosphate.

The separation of true inorganic and labile phosphates, and their concomitant determination, were effected by the following procedure: The sample solution was adjusted to contain 5 to 10 micromoles of total true inorganic and labile phosphates in 1 ml. of solution at pH 6.5. 1 ml. of this solution was placed in a 15 ml. conical centrifuge tube. To it were added 1 ml. of ammonium acetate buffer reagent (a solution 0.5 M with respect to ammonium hydroxide and 0.05 M with respect to $NaHCO_3$, adjusted to pH 8.5 with acetic acid) and 4 ml. of 1.0 M calcium chloride in 95 per cent ethyl alcohol. The resulting precipitate of calcium carbonate and inorganic phosphate was allowed to flocculate for 5 to 7 minutes. The tube was then centrifuged for about 5 minutes. The supernatant liquid was poured off, and the precipitate was washed on the centrifuge with 2 ml. of alcoholic calcium chloride solution. All of the above operations were carried out in the cold (0–5°) to minimize hydrolysis of acetyl phosphate. The precipitate was dissolved with a drop of concentrated hydrochloric acid and diluted to a suitable volume. In this solution inorganic phosphate was determined by the Fiske and Subbarow method as described; the value thus obtained was assumed to represent the content of true inorganic phosphate in the sample. At the same time, the Fiske and Subbarow method was applied directly to a suitable aliquot of the original sample solution; the value thus obtained was assumed to represent the sum of the true inorganic plus labile phosphate content in the sample solution. Labile phosphate content was then calculated by difference.

Several experiments were conducted to check the accuracy of this separation procedure. The observation by Lipmann[2] that the coprecipitation of calcium carbonate is necessary for complete precipitation and flocculation of calcium phosphate was verified. It was found that inorganic phosphate was precipitated 97 to 100 per cent under the conditions given. In order to determine the action of labile phosphate in this course of procedure, a sample of disilver acetyl phosphate[3] was converted to the sodium salt by shaking in the cold with sodium chloride solution. It is probable that some hydrolysis of the acetyl phosphate occurred during the process. The results of recoveries on this solution are given in Table II. When applied to enzyme reaction mixtures, experience indicated that this separation procedure gave slightly low values for labile phosphate if the ratio of inorganic phosphate to labile phosphate was greater than 4.0, and slightly high values if the ratio was less than 0.3. In the latter case and in other cases when advisable, known quantities of inorganic phosphate were added to the sample to bring the ratio to 1.0 to 1.3 before the assay was repeated.

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[2] Private communication.

[3] We are indebted to Fritz Lipmann for generous gifts of disilver acetyl phosphate, and for helpful advice in this work.

The 7 minute and stable ester phosphates were determined as follows: By calculation from the amount of inorganic phosphate originally added to the reaction mixture, and the amount of true inorganic and labile phosphate found in the mixture after reaction, the approximate amount of 7 minute plus stable ester phosphate in the sample was determined. The sample was diluted to contain approximately 10 micromoles of total phosphates in 1 ml. of solution at pH 6.5. To 1 ml. of this solution, 1 ml. of 2.0 N hydrochloric acid was added, and the solution was placed in a steam bath for exactly 7 minutes and cooled. Inorganic phosphate was then determined in this solution by the modified Fiske and Subbarow method described previously. 7 minute phosphate was calculated by subtracting the sum of labile plus inorganic phosphate, previously determined, from the inorganic phosphate found in this determination. Stable phosphate was calculated by subtracting the sum of inorganic, labile, and 7 minute phosphates from the amount of inorganic phosphate originally added to the reaction mixture.

TABLE II

Recovery of Acetyl Phosphate in Presence of Inorganic Phosphate

The sample contained the indicated amounts of added inorganic phosphate and acetyl phosphate preparation in 1 ml. at pH 6 5.

Inorganic phosphate added	Total phosphate added as acetyl phosphate preparation	Total phosphate pptd.	Labile phosphate in acetyl phosphate preparation
micromoles	*micromoles*	*micromoles*	*per cent*
8.0	1.76	8.40	77.3
6.0	3.52	6.88	75.0
5.0	4.40	6.20	72.7
4.0	5.28	5.40	73.5
2.0	7 03	4.07	70.5
0.0	8.80	2.62	70.2

Determination of Silver-Precipitable Volatile Acids—This procedure was applied to enzyme reaction mixtures after protein precipitation with trichloroacetic acid and neutralization or to purified labile phosphate solutions. The sample was adjusted to contain 16 to 20 micromoles of labile phosphate in 2.0 ml. of solution at pH 6.5. To 2.0 ml. of this solution, 13 ml. of 95 per cent ethyl alcohol and 200 micromoles of silver nitrate in 0.2 ml. of solution were added. The resulting precipitate was centrifuged out, washed twice with 4 ml. portions of 95 per cent ethyl alcohol, and taken up in 5.0 ml. of 0.1 N sulfuric acid. This suspension was steamed for 15 minutes in a stoppered tube, cooled, diluted to 30 ml., and subjected to Duclaux distillation.

For this distillation the entire hydrolysate was placed in an all-glass distilling apparatus. The first 5.00 ml. of distillate, containing carbon dioxide, was discarded. The two succeeding 10.00 ml. portions were heated just to a boil, cooled rapidly, and titrated to a phenol red end-point with standard 0.01 N barium hydroxide solution. The content of acetic and butyric acids in the sample was then calculated by the usual method (4). The rate of distillation was adjusted so that, after rejection of the first 5 ml. of distillate, the following 10 ml. portion was delivered in 7.5 minutes. Before analyses of samples, the apparatus was calibrated with standard solutions of acetic and butyric acids under the same conditions, and consistently gave recoveries of 96 to 100 per cent on mixtures of these two acids.

At all times solutions believed to contain acetyl phosphate were kept cold, in an ice bath or preferably frozen, when possible, to minimize hydrolysis.

Phosphate Balances in Presence and Absence of Glucose—In earlier work, attempts to detect the formation of a stable phosphorylation product in pyruvic acid fermentations by cell-free extracts of *Clostridium butylicum* had failed. Apparently any labile phosphates produced had been hydrolyzed during the determination of inorganic phosphate, and had escaped detection. However, application to fermented liquors of the above method for concomitant determination of labile and true inorganic phosphate indicated that labile phosphate was indeed produced, at the expense of inorganic phosphate. Lipmann reported that enzyme preparations used in this work would also catalyze the phosphorylation of added glucose during pyruvic acid fermentation, with the accumulation of stable ester phosphate. The magnitude of these reactions was studied in the following phosphate balances of pyruvic acid fermentation in the presence and absence of glucose.

The reaction was carried out in the usual manner (1). After reaction, the contents of the flasks were washed into 15 ml. conical centrifuge tubes and diluted to 5 ml. with water. Proteins were precipitated by the addition of 1 ml. of 1.0 M trichloroacetic acid to each tube. After immediate centrifugation, the supernatant liquids were rapidly adjusted to pH 6.5 with 1 N sodium hydroxide solution and diluted to 10 ml. On these solutions the indicated determinations were made. The results are given in Table III.

In the absence of glucose, inorganic phosphate was taken up and appeared as labile phosphate esters. In the presence of glucose, the reaction rate increased 133 per cent; inorganic phosphate was again taken up, but appeared as stable ester phosphate plus 7 minute phosphate instead of as labile phosphates. Because the esters in this case were probably a mixture of hexose phosphate esters whose character is not of great importance to the present study, they were not characterized further.

Indications of Character of Labile Phosphate—As mentioned previously (1), the fermentation of pyruvic acid by cell-free extracts of *Clostridium butylicum* appears to be somewhat similar to the oxidation of pyruvic acid catalyzed by enzyme systems prepared by Lipmann (5) from cells of *Lactobacillus delbrueckii.* The discovery that labile phosphate is formed in the former reaction leads naturally to the supposition that acetyl phosphate, isolated by Lipmann (6) in the oxidation reaction, is also an intermediate labile phosphate ester in the fermentation reaction.

The isolation of labile phosphate from fermentation mixtures for rigid characterization was attempted and was unsuccessful. Pyruvic acid dissimilation by cell-free extracts under the conditions described previously (1) is slow, and the amounts of labile phosphate obtained are too small and solutions are too dilute for such isolation; furthermore, labile phosphate is relatively unstable at incubation temperature. In order to force reasonably rapid reaction, it was necessary to use great excesses of inorganic phosphate in the reaction mixtures. The resulting problem of separating the excess inorganic phosphate from labile phosphate proved difficult. Precipitation of inorganic phosphate with calcium nitrate in the concentrated reaction mixtures which were obtained resulted in coprecipitation and loss of much labile phosphate, and purified solutions of labile phosphate hydrolyzed with such ease that satisfactory separation from inorganic phosphate could not be effected.

TABLE III

Phosphate Balance of Pyruvic Acid Fermentation in Presence and Absence of Glucose

The Warburg flasks contained 97 micromoles of inorganic phosphate and 30 mg. of dry enzyme powder; pH 6.5. Total volume, 2.8 ml. KOH in the center cup. Reaction time, 60 minutes. All values are expressed in micromoles.

Flask No.	Pyruvate added	Glucose added	Hydrogen evolved	Inorganic phosphate	Labile phosphate	7 minute phosphate	Stable phosphate
1	0	0	−0.4	94	2	3	−2
2	0	0	−0.2	94	3	4	−4
3	50	0	14.4	81	14	4	−2
4	50	0	14.2	81	13	7	−4
5	0	150	−0.3	92	2	2	1
6	0	150	−0.3	91	3	1	4
7	50	150	34.9	53	3	11	30
8	50	150	31.4	54	3	11	29

However, indications of the character of the labile phosphate were secured by precipitating purified labile phosphate solutions with silver, regenerating the sodium salts, and hydrolyzing these with acid. The volatile acid content of the hydrolysates was then correlated with the labile phosphate content of the sodium salt solution. It was shown that added volatile acids were not precipitated with silver under these conditions, and that no volatile acids could be detected in analogous silver precipitates from blank reaction mixtures in which no fermentation had taken place.

To obtain labile phosphate for this purpose, pyruvic acid fermentation was carried out in the usual manner, but in a Warburg flask of about 115 ml. capacity. The manometer was filled with mercury. The reaction mixture consisted of 4000 micromoles of inorganic phosphate, 800 micromoles of sodium pyruvate, and 500 mg. of dry enzyme powder, at pH 6.5 in a volume of 19 ml. KOH was placed in the center cup. After 140 minutes incubation the contents of the flask were removed and diluted with washing to 23 ml. To precipitate proteins, 3 ml. of 1.0 M trichloroacetic acid were added, and the suspension was immediately centrifuged. The supernatant liquid was adjusted to pH 6.7 with 1 M sodium hydroxide solution. The total volume of 32 ml. contained 2820 micromoles of inorganic phosphate and 280 micromoles of labile phosphate.

To remove inorganic phosphate, 3000 micromoles of calcium nitrate in 3 ml. of solution were added, and the pH was readjusted to 6.5 with 1 N sodium hydroxide solution. The resulting calcium phosphate precipitate was centrifuged out. Much labile phosphate had coprecipitated. The volume was adjusted to 19 ml. This solution contained 123 micromoles of labile phosphate and 63 micromoles of inorganic phosphate. To 15 ml. of this solution 60 ml. of 95 per cent ethyl alcohol and 550 micromoles of silver nitrate in 1.1 ml. of solution were added. The resulting silver precipitate was washed once with 10 ml. and once with 6 ml. of alcohol. The precipitate was suspended in 10 ml. of 0.044 N sodium chloride solution (containing one-thirtieth of the chloride ion as hydrochloric acid in order to attain pH 6.5 to 7.0 after the cation exchange) and shaken well. The suspension was centrifuged, and the supernatant liquid containing sodium salts of labile phosphate at pH 7.0 was diluted to 12 ml. Assay indicated that this solution contained 59 micromoles of labile phosphate and 22 micromoles of inorganic phosphate. To 6.7 ml. of this solution (33 micromoles of labile phosphate) 205 micromoles of silver ion in 5.7 ml. of silver sulfate solution and 5.0 ml. of 0.1 N sulfuric acid solution were added. The resulting suspension was steamed in a stoppered tube for 10 minutes to hydrolyze the labile phosphates and cooled. The precipitate of silver chloride was centrifuged out; the supernatant liquid gave no further precipitate when tested with a drop of silver sulfate solution. The supernatant liquid was diluted to 40 ml. and subjected to Duclaux distillation.

The sample was adjusted to contain 30 to 40 micromoles of volatile acids, 36 micromoles of silver ion as silver sulfate, and 5.0 ml. of 0.1 N sulfuric acid, in 40 ml. of solution. After 5 ml. of distillate were discarded, three (instead of two) succeeding 10 ml. portions were titrated as before. The apparatus had been previously calibrated with standard solutions of acetic, butyric, valeric, and isovaleric acids under identical conditions.

The titration values obtained in the distillation of the labile phosphate hydrolysate did not conform to the volatility of acetic acid alone; approximately one-third of the volatile acid present was more volatile than acetic acid. Since butyric acid is a normal product of glucose dissimilation by *Clostridium butylicum*, it was believed that butyric acid might also be present. Therefore, the distillation results were recalculated on the assumption that the volatile acid distilled was a binary mixture of acetic and butyric acids. Results of separate calculations involving all possible combinations of two titration values (4) agreed well. Similar calculations based on the assumption that a binary mixture of acetic and of several other more volatile acids was present disagreed widely. The results of these calculations are given in Table IV.

TABLE IV

Identification of Silver-Precipitable Volatile Acids by Duclaux Distillation

Results of Duclaux distillation calculated from titratable acidities of Fractions I, II, and III for the following combinations of volatile aliphatic acids.

Acids assumed present		Micromoles of acid in Ag ppt calculated from		
		Fractions I and II	Fractions I and III	Fractions II and III
Acetic and butyric	Acetic acid	23.6	24.7	24.3
	Butyric "	12.4	12.1	12.0
	Total	36.0	36.8	36.3
Acetic and valeric	Acetic acid	31.7	28.6	27.9
	Valeric "	7.7	9.2	12.0
	Total	39.4	37.8	39.9
Acetic and isovaleric	Acetic acid	34.1	29.3	28.9
	Isovaleric acid	6.4	8.5	13.5
	Total	40.5	37.8	42.4

It was, therefore, assumed that the mixture of volatile acids in the labile phosphate hydrolysate consisted chiefly of acetic and butyric acids. Thus the aliquot containing 33 micromoles of labile phosphate also contained, after hydrolysis, 24.2 micromoles of acetic acid and 12.2 micromoles of butyric acid.

To show that the presence of silver-precipitable acetic and butyric acids in the purified labile phosphate preparations was associated with the fermentation of pyruvic acid and that such acids were not present before fermentation, a control experiment was performed. A blank enzyme reaction mixture was prepared containing 2000 micromoles of inorganic phosphate, 400 micromoles of acetic acid, and 250 mg. of dry enzyme powder, at pH 6.5 in 9.5 ml. of solution. This reaction mixture was intended to simulate the fermented enzyme reaction mixture from which the labile phosphate solutions in previous experiments had been prepared. Since no fermentation had occurred in this control mixture, there should have been no accumulation of labile phosphate, and no acetic or butyric acids should be found in analogous silver precipitates. On this mixture an exactly analogous procedure for preparing labile phosphate solution was carried out. Both the labile phosphate and silver-precipitable volatile acid contents of this solution were determined. None of either could be detected.

Silver acetate and butyrate are sparingly soluble in water; thus it might be possible that these salts were merely precipitated as such from the purified labile phosphate preparations, since no separation of labile phosphate from acetic and butyric acids originally occurring in the fermented reaction mixture had been made. That any acetic acid present was not precipitated under the conditions existing during the silver precipitation has been shown by the control experiment just described. That any butyric acid present similarly was not precipitated was shown by a second experiment. A labile phosphate solution was prepared as has been described. A 2 ml. aliquot of this solution had been found to contain 18.5 micromoles of labile phosphate, 11.23 micromoles of silver-precipitable acetic acid, and 4.90 micromoles of silver-precipitable butyric acid. To a second 2 ml. aliquot, 10 micromoles of sodium butyrate in 0.2 ml. of solution at pH 6.5, and to a third 2 ml. aliquot 0.2 ml. of water were added. The solutions were assayed for silver-precipitable volatile acids. It was found that in the second aliquot 8.90 micromoles of acetic acid and 5.03 micromoles of butyric acid, and in the third aliquot 9.36 micromoles of acetic and 4.98 micromoles of butyric acids, were precipitated with silver. The difference between these values is within experimental error, and it was therefore assumed that none of the added butyric acid had been precipitated.

These experiments indicate that the acetic and butyric acids precipitated with silver from purified labile phosphate solutions were not present as such, that their presence is related to the production of labile phosphate in the pyruvic acid fermentation reaction, and that they may be present as acetyl and butyryl phosphates.

Source of Silver-Precipitable Butyric Acid—After the indication that acetyl and butyryl phosphates may be formed in the fermentation reaction was established, the chemical mechanism by which silver-precipitable butyric acid arises was studied. The enzyme preparation had a pronounced butyric acid odor; a transphosphorylation reaction between butyric acid contained in the enzyme preparation and acetyl phosphate formed in the reaction, to yield butyryl phosphate and acetic acid, was postulated. This reaction would yield acetic acid and butyryl phosphate.

This possibility was investigated in the experiment outlined in Table V. In the presence of enzyme extract, acetyl phosphate was incubated both with (Flask 1) and without (Flask 2) added butyric acid. In the control (Flask 3), acetyl phosphate was not added to the reaction mixture until after the enzyme preparation had been inactivated; this procedure was intended to prevent the endogenous transphosphorylation evident in Flask 2. The initial reaction mixtures given in Part I of the table were incubated in Warburg flasks as described. After incubation the contents of each flask

TABLE V

Source of Silver-Precipitable Butyric Acid

All solutions adjusted to pH 6.5 before addition. Gas phase, hydrogen. Temperature, 37°. Reaction begun by tipping enzyme suspension from side arm into main compartment. Incubation time, 30 minutes. No KOH in the center cup.

	Flask 1 Active		Flask 2 Endogenous		Flask 3 Control	
Part I. Initial additions						
Volume of reaction mixture	3.3 ml.					
Sodium acetyl phosphate	150 μM		150 μM		0 μM	
" butyrate	150 "		0 "		150 "	
Enzyme preparation	100 mg.		100 mg.		100 "	
Part II. Additions after protein pptn. with trichloroacetic acid						
Sodium acetyl phosphate	0 μM		0 μM		150 μM	
" butyrate	0 "		150 "		0 "	
Water	Sufficient to make up to 7.0 ml.					
Part III. Results						
	micromoles		*micromoles*		*micromoles*	
Gas evolved (as CO_2)	+2.07		+0.44		+5.31	
Phosphate distribution*						
Inorganic phosphate	59.9	60.0	57.5	61.8	32.2	32.3
Labile phosphate	82.0	81.0	78.5	74.5	104.2	104.1
Volatile acids in silver ppt.*						
Acetic acid	34.6	37.8	45.4	45.7	89.0	91.3
Butyric "	25.1	23.8	13.3	13.1	−1.2	−1.4
Total volatile acids	59.7	61.6	58.7	58.8	87.8	89.9
Micromoles volatile acid per micromoles labile P	0.733	0.756	0.765	0.767	0.848	0.862

* Duplicate determinations are given.

were removed and diluted to 5.0 ml. by washing. Proteins were precipitated and enzymes were inactivated by adding 1 ml. of 1.0 M trichloroacetic acid. At this time, before the resulting precipitate was centrifuged out, the additions indicated in Part II were made. These additions were intended to produce the same concentrations of butyric acid and labile phosphate in all of the reaction mixtures, so that subsequent silver precipitations would occur under comparable conditions in active and control samples; however, because the acetyl phosphate in the enzyme-containing reaction mixtures of Flasks 1 and 2 was subjected to incubation temperature and the action of phosphatases during incubation, while the acetyl phosphate now added to the reaction mixture of Flask 3 had been kept cold and separate, the labile phosphate level in Flask 3 was higher during the silver precipitation than the level in Flasks 1 and 2. The protein precipitates were immediately removed by centrifuging, and the supernatant liquids were adjusted to pH 6.5 with 1 N sodium hydroxide solution. The solutions were diluted to 8.0 ml. and assayed for labile phosphate and silver-precipitable volatile acids.

The results of this experiment are given in Part III of Table V. No butyric acid was found in the silver precipitate from the reaction mixture in which the enzyme preparation was inactivated before the addition of acetyl phosphate, and the addition of butyric acid to the incubated reaction mixture resulted in a large increase in the amount of silver-precipitable butyric acid. These results indicate that an exchange of the phosphate group of acetyl phosphate and butyric acid had taken place.

It might be assumed that the butyryl phosphate was produced by condensation and reduction of 2 acetyl phosphate molecules. In the above experiment in which acetyl phosphate was incubated with enzyme preparation under molecular hydrogen, this would have been indicated by gas uptake during incubation. Instead there was slight gas evolution. Therefore, unless the necessary reductive hydrogen was provided by a suitable hydrogen donor in the enzyme preparation, such condensation and reduction did not occur.

DISCUSSION

Intermediate phosphorylation apparently occurs in the fermentation of pyruvic acid by cell-free preparations of *Clostridium butylicum*. Inorganic phosphate is taken up in the reaction, and labile phosphate appears. In the presence of glucose, stable ester phosphate is produced in place of labile phosphate. The demonstration of this series of intermediate phosphorylations suggests that fermentative energy utilization in this butyric acid anaerobe is in accord with the current concept (7, 8) that the energy of carbohydrate dissimilation by living cells is utilized by means of the generation and hydrolysis of high energy phosphate bonds.

The data of Table III and of the previous paper (1) are consistent with the assumption that the reaction taking place is

$$\text{Pyruvate}^- + H_2PO_4^- \rightleftharpoons CO_2 + H_2 + \text{acetyl phosphate}^=$$

In the presence of compounds (such as butyric acid or glucose) capable of functioning as phosphate acceptors, a transphosphorylation occurs. From the estimations made by Kalckar (7) it is apparent that the decomposition of pyruvate into acetate, carbon dioxide, and hydrogen involves, at pH 7 and when reactants and products are present at equal concentrations, a free energy change of approximately 10,000 calories. Although the free energy of formation of acetyl phosphate is not accurately known, generally accepted values for the energy of a carboxyl phosphate bond are also in the neighborhood of 10,000 calories. The experimental data also indicate that we are dealing with an over-all reaction involving very little energy change. A relatively high concentration of inorganic phosphate must be present in order to obtain a satisfactory reaction rate. The reaction rate is also much increased when glucose is the ultimate phosphate acceptor. (The phosphate bond energy in hexose phosphates is much less than that in carboxyl phosphates.)

Both of these observations suggest that the equilibrium position of the reaction is such that maximum reaction velocity in a positive direction is obtained only when reactant concentration is much higher than product concentration.

Rigid characterization of the labile phosphate produced in the pyruvic acid fermentation reaction has not been achieved because of inability to isolate the labile phosphorus compound. However, indications of the presence of acetyl and butyryl phosphates have been obtained. These are as follows: Purified labile phosphate solutions from pyruvic acid fermentation reaction mixtures contain amounts of silver-precipitable acetic and butyric acids which correlate reasonably with the labile phosphate content of the solutions. Similar solutions obtained from blank fermentation reaction mixtures in which no fermentation has taken place do not contain silver-precipitable volatile acids. Added acetic and butyric acids are not precipitated by the silver treatment. Finally, incubation of acetyl phosphate with butyric acid in the presence of enzyme extract results in the formation of silver-precipitable butyric acid in the reaction mixture.

The apparent ability of the enzyme preparation to catalyze transphosphorylation between acetyl phosphate and butyric acid to produce butyryl phosphate suggests that butyryl phosphate may play an intermediary rôle in the production of butyric acid and butyl alcohol by *Clostridium butylicum.*

The production of labile phosphate, apparently acetyl phosphate, is a point of similarity between pyruvic acid fermentation by preparation of *Clostridium butylicum* to acetic acid, carbon dioxide, and hydrogen, and by preparations of *Escherichia coli* to acetic and formic acids (9) and pyruvic acid oxidation by preparations of *Lactobacillus delbrueckii* (5).

SUMMARY

1. The preparation from *Clostridium butylicum* of a large supply of cell-free dried water extract of frozen cells which catalyzes pyruvic acid fermentation is described. 36 per cent of the activity of the frozen cells was recovered in the dried extract.

2. Phosphate balances of the fermentation of pyruvic acid by enzyme extract in the presence and absence of glucose are given. In the absence of glucose, inorganic phosphate is taken up and appears as labile phosphate, but no stable phosyphorylation product accumulates. In the presence of glucose, no labile phosphate accumulates, but inorganic phosphate is taken up and appears as stable ester phosphate.

3. Attempts to isolate labile phosphate from the fermentation mixture were unsuccessful. Silver-precipitable acetic and butyric acids were present in purified labile phosphate preparations. Apparently the labile phosphate is a mixture of acetyl and butyryl phosphates.

4. Silver-precipitable butyric acid, apparently butyryl phosphate, arises by incubation of acetyl phosphate with butyric acid in the presence of enzyme extract. An exchange of the phosphate group of acetyl phosphate apparently occurs between acetyl phosphate and butyric acid to yield acetic acid and butyryl phosphate.

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HYDROXYLEUCINES

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(Received for publication, April 15, 1944)

The following paper is chiefly concerned with the synthesis of γ-hydroxyleucine and the description of some of its derivatives. The immediate reason for interest in this amino acid is as follows. In the course of fractionating, under conditions referred to below,[1] a quantity of amino acids from casein, a small amount of a substance was fortuitously isolated which appeared to be homogeneous and which could not be identified with any known amino acid. Analysis consistently indicated the formula $C_6H_{13}NO_3$

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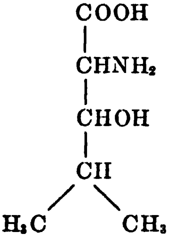
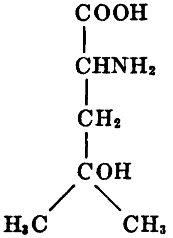
[1] The following notes regarding the unidentified substance are obviously incomplete and are only recorded in the hope that they may possibly be of use to later workers. In the course of fractionating a mixture of monoamino acids and a larger proportion of dicarboxylic amino acids from casein, so called "pure" commercial casein purchased from Eimer and Amend, use was made of precipitation with silver nitrate and sodium hydroxide. On decomposition of the sparingly soluble silver salts with hydrogen sulfide and concentration of the filtrate under diminished pressure, the product was found to contain significant amounts of nitrate. The vacuum-dried product was repeatedly extracted with alcohol which dissolved most of the nitrate in combination with monoamino acids. The nitrate was removed by precipitation of the amino acids with mercuric acetate and sodium carbonate. The amino acids were then converted into copper salts and a little phenylalanine was removed as the sparingly soluble salt. On removal of copper from the soluble salts and concentration, crystallization slowly took place. The crude product, only about 3 gm. from 2.5 kilos of casein, still contained a trace of phenylalanine which was eventually removed by repeated recrystallization from aqueous alcohol. (Evaporation with concentrated nitric acid followed by treatment with ammonia gives a strong orange-yellow color if phenylalanine or tyrosine is present.) The melting point of the repeatedly recrystallized substance was 248–250°; it dissolved in less than half its weight of hot water and was insoluble in alcohol. Three analyses of different preparations fell within the following limits, C 48.3 to 49.1, H 8.40 to 8.80, N 9.63 to 9.77. Calculated for $C_6H_{13}NO_3$, C 49.0, H 8.84, N 9.52. Amino nitrogen (Van Slyke) 9.60. Feebly levorotatory in aqueous solution changing to dextro on addition of HCl. Periodate oxidation negative, iodoform negative, traces of acetone with $K_2Cr_2O_7$; copper salt clear blue and very soluble; Reinecke, flavianic acid, naphthalene-β-sulfonic acid, and picric acid all negative. Phosphotungstic acid yields a precipitate immediately in 0.3 per cent solution and slight precipitation on standing in 0.2 per cent. Reduction with hydriodic acid and phosphonium iodide at 140° gave *l*-leucine showing dextrorotation in acid solution. $C_6H_{13}NO_2$ calculated, C 55.0, H 9.92, N 10.7; found, C 55.2, H 9.8, N 10.3. The leucine was converted into the uramino acid, m.p. 214°, showing no depression of melting point when mixed with an authentic specimen from *l*-leucine. Up to the present, efforts to simplify the preparation of additional quantities of the amino acid have had scant success.

and the whole of the nitrogen was in the amino form. The substance was neutral in reaction and formaldehyde titration indicated one carboxyl group. No evidence of a lactone grouping could be obtained, so that the inference seemed clear that a hydroxyamino acid was present. On reduction with hydriodic acid (sp. gr. 1.96) and phosphonium iodide at 140°, an amino acid $C_6H_{13}NO_2$ was obtained in about 50 per cent yield and was identified as *l*-leucine. This in turn was converted into the uramido acid and found identical in composition and mixed melting point with the substance prepared from authentic *l*-leucine, thus definitely excluding the possibility of a derivative of *nor*- or *iso*leucine, which melt at much lower temperatures. The evidence thus far conformed to the view that the unknown substance was a hydroxyleucine. On oxidation with periodic acid as described by Van Slyke, Hiller, and MacFadyen (1) practically none (0.2 per cent) of the nitrogen was liberated as ammonia, so that the β position for the hydroxy group (I) appeared ruled out, leaving the γ-hydroxyleucine (II) and δ-hydroxyleucine (III) still to be considered. The synthesis of *dl*-γ-hydroxyleucine is described in the following pages and it may be stated at once that,


(I)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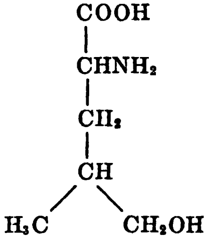
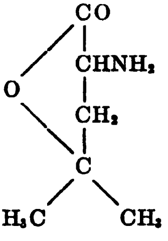

(III)

while it shows many resemblances to the unknown amino acid, the differences are too great to support the view that it represents the optically inactive form of the active natural amino acid. For example the melting point of γ-hydroxyleucine is 226–228° compared with 248–250° for the natural acid. Flavianic acid gives a sparingly soluble salt with γ-hydroxyleucine, but the flavianate of the natural acid is extremely soluble. The copper salt of γ-hydroxyleucine is only moderately soluble in cold water and crystallizes in long blue needles with a marked violet tinge, while the copper salt of the natural acid is pure blue in color and is very soluble in water. Reinecke acid gives a rather soluble reineckate of γ-hydroxyleucine crystallizing in long needles, while the amino acid from casein gives no precipitate. Naphthalene-β-sulfonic acid gives a fairly soluble crystalline nasylate with γ-hydroxyleucine but no precipitate with the natural amino acid.

The behavior of the two amino acids with phosphotungstic acid has some points of interest. It may be recalled that Sörensen (2) showed that α-amino-δ-hydroxy-*n*-valeric acid was slowly precipitated by phosphotungstic acid, while Fischer and Zemplén (3) found that δ-amino-α-hydroxyvaleric acid showed the same behavior and surmised that slow conversion of the amino acid into β-hydroxypiperidone was a necessary part of the reaction. On addition of phosphotungstic acid to even a concentrated solution (5 per cent) of γ-hydroxyleucine, there is no immediate precipitate but after some minutes large exceptionally well formed glistening needles separate. The same finely crystalline phosphotungstate is obtained from solutions as dilute as 0.3 per cent but only after standing several days. On the other hand the amino acid from casein gives an immediate oily precipitate, in concentrations of 0.3 per cent and greater, which on standing is converted into small needles. One further difference between the two amino acids may be cited. On destructive distillation, the natural amino acid yields vapors giving a strong pyrrole reaction, whereas γ-hydroxyleucine gives a virtually negative result.

It is obvious from the foregoing that the synthesis of δ-hydroxyleucine for comparison with the natural acid is highly desirable. Some few attempts have been unsuccessful and, while the writer hopes to continue these experiments, he expressly disavows any possible claim to priority.

In view of the extreme ease with which γ-hydroxyisocaproic acid is converted into isocaprolactone even at room temperature (4), it is interesting to note that γ-hydroxyleucine does not readily yield α-aminoisocaprolactone (IV) either in hot aqueous solution or on boiling with hydrochloric acid. On the other hand the phenyl isocyanate derivative of γ-hydroxyleucine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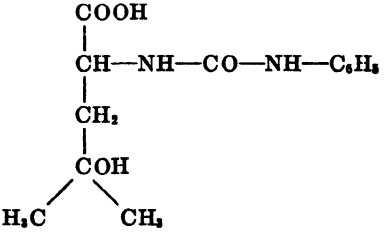
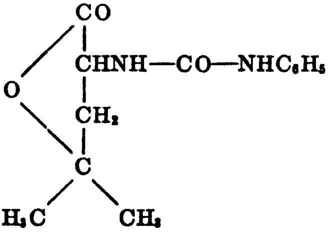
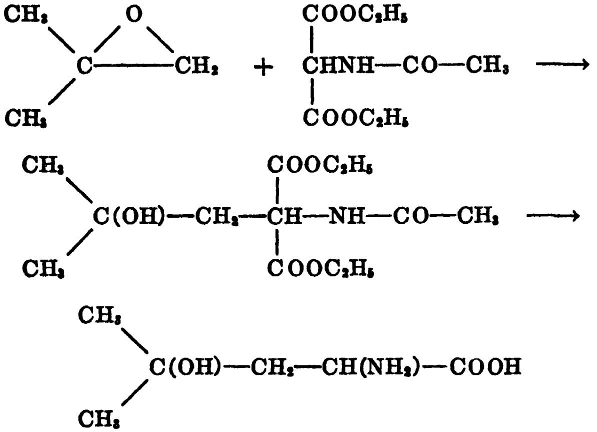

(V)


(VI)

(V) is promptly converted into its lactone (VI). Whether the preceding facts constitute evidence in favor of the zwitter ion theory of the structure of α-amino acids according to which the carboxyl group would be less available for lactone formation is a matter of speculation.

EXPERIMENTAL

The method finally adopted for the synthesis of γ-hydroxyleucine consists in the condensation of isobutylene oxide and acetylaminomalonic diethyl ester, followed by hydrolysis, as shown in the accompanying reactions. Traube and Lehmann (5) had already investigated the condensation of unsubstituted malonic ester with ethylene oxide and with epichlorhydrin, the product from the latter serving as starting material for Leuch's synthesis of hydroxyprolines. The condensation of acylaminomalonic esters with alkylene oxides, so far as the writer knows, has not been previously investigated and should prove a useful method for the synthesis of γ-hydroxy-α-amino acids.



A few preliminary experiments showed that the condensation proceeded unsatisfactorily under the conditions chosen by Traube and Lehmann, which consisted of adding a molecular proportion of an alcoholic sodium ethylate solution to the ester, followed by addition of the alkylene oxide to the resulting sodium malonic ester. Nor was the use of smaller amounts of sodium ethylate as a catalyst any more successful. Further experiments showed that dry sodium methylate with or without the use of dioxane as diluent gave satisfactory results, although there is no reason to believe that the conditions to be described are not capable of further improvement. Acetylaminomalonic ester[2] (43.4 gm. = 0.2 mole) in a round bottom flask with attached reflux condenser is mixed with excess of isobutylene oxide, at least twice and preferably three times the theoretical amount, diluted with an equal volume of dry dioxane. Dry sodium methylate (0.4 mole) is then added in four divided portions at hourly intervals while the contents of the flask are occasionally shaken and gently simmered over a water bath. Heating is continued for 6 to 8 hours. Water (50 cc.) is then added, followed by the cautious addition of hydrochloric acid (200 cc.). Hydrolysis is effected by boiling the mixture under a reflux for 16 to 20 hours in an oil bath. On cooling, the solution is filtered to remove a minute amount of oil and then concentrated under diminished pressure to remove as much hydrochloric acid as possible. The residue is then treated with warm alcohol, the residual sodium chloride filtered off, and the filtrate again concentrated under diminished pressure, leaving a finely crystalline residue. It is desirable to remove the sodium chloride as completely as possible, so that a second treatment of the residue with absolute alcohol is of advantage. After removal of alcohol, the residue is dissolved in water and treated in one of two ways. In the first of these the solution is boiled with excess of freshly precipitated lead hydroxide to remove the bulk of hydrochloric acid. The small amount of chloride remaining in the filtrate is removed by the cautious addition of silver acetate. After the material is filtered again, lead and silver are removed with hydrogen sulfide and the filtrate is concentrated under diminished pressure. The concentrated solution on slow evaporation is almost entirely converted into crystalline amino acid which is washed with alcohol before recrystallization. This method gives a larger yield of amino acid than the one to be next described but, unfortunately, the product is usually contaminated with more or less glycine derived from the hydrolysis of unchanged acetylaminomalonic ester. Since glycine is slightly less soluble in both water and aqueous alcohol than γ-hydroxyleucine, recrystallization of the product is usually of little advantage.

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[2] For generous supplies of acetylaminomalonic ester I am indebted to Merck and Company, Inc., through the kind cooperation of Dr. R. T. Major and Dr. E. E. Howe.

It has already been stated that γ-hydroxyleucine gives a sparingly soluble flavianate and use has been made of this for the separation of the amino acid. The aqueous solution of the hydrochloride obtained as just described is precipitated with a concentrated solution of about 80 gm. of flavianic acid, when on cooling crystallization takes place rapidly. After 48 hours in the ice box the soft crystals are filtered off and washed with ice water. They are then dissolved in hot water and decomposed by the addition of barium hydroxide dissolved in hot water. The barium hydroxide is added until a test portion reddens phenolphthalein paper, about 70 gm. being required. The barium flavianate is filtered off and well washed. The slight excess of barium in the filtrate is quantitatively removed with sulfuric acid, and a little decolorizing carbon is added to remove the residual trace of flavianic acid. On concentration to small bulk, the γ-hydroxyleucine separates out as large transparent nodular masses of needles which become opaque on drying. The yield of once crystallized product amounts to about 50 per cent of the theoretical, that is from 14 to 16 gm. The substance was recrystallized for analysis by dissolving it in half its weight of hot water and adding several volumes of methyl alcohol. It separated as fine silky needles and was dried at 75° in a vacuum over phosphorus pentoxide.

$C_6H_{13}NO_3$. Calculated. C 49.0, H 8.84, N 9.52
Found. " 48.8, " 8.85, " 9.61

The amino nitrogen by Van Slyke's method was 9.65. The amino acid, after sintering slightly at about 220°, melts sharply at 226–228° with vigorous effervescence, yielding a clear yellow-brown melt.[3] The copper salt was prepared in the usual way by boiling an aqueous solution of the amino acid with copper carbonate. It crystallized very easily in exceptionally attractive long deep blue needles which on drying acquired a definite violet tinge. It is moderately soluble in cold water but very easily soluble in hot water and has the normal composition. The air-dried salt contains no water of crystallization.

$(C_6H_{12}O_3N)_2Cu$. Calculated, Cu 17.9, N 7.87; found, Cu 17.8, N 7.85

The preparation of the flavianate has already been referred to. It is freely soluble in hot water and on cooling separates as fine soft lemon-yellow needles melting at 272–273°.

$C_{10}H_6O_8N_2S \cdot C_6H_{13}NO_3$. Calculated. C 41.7, H 4.12, N 9.11
Found. " 42.0, " 3.86, " 9.20

The phenyl isocyanate derivative of γ-hydroxyleucine was prepared in the usual way. When the alkaline solution was acidified, precipitation was immediate and the product was recrystallized from a mixture of methyl alcohol and water. It crystallizes in needles and melts at 188–189° with slight previous softening. The analysis indicates that the substance is not a simple addition compound $C_{13}H_{18}N_2O_4$ requiring C 59.9 and H 6.77, but that a molecule of water has been eliminated presumably through lactone formation. The action of hot hydrochloric acid on the compound is complicated and yields several products which have not been closely characterized.

$C_{13}H_{16}O_3N_2$. Calculated, C 62.9, H 6.45; found, C 63.0, H 6.59

The precipitation of the phosphotungstate of γ-hydroxyleucine and also the reineckate melting at 167–170° have already been referred to and need no further comment. The amino acid does not give more than a very slight iodoform reaction. Oxidation with potassium dichromate gives some acetone. Oxidation with periodic acid in alkaline solution gives virtually no ammonia (0.2 per cent of N), thus confirming the γ position of the hydroxyl group.

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[3] Any contamination of γ-hydroxyleucine with glycine is easily recognized by the much darker color of the melt.

Glycine Flavianate—In some of the earlier experiments on the preparation of γ-hydroxyleucine in which use was made of flavianic acid for its precipitation, it was noted that on occasion, after the material had stood for some time, a flavianate separated with markedly different properties from those of the hydroxyleucine flavianate. It crystallized as a light lemon-yellow hard crust of needles and was identified as glycine flavianate. This seemed surprising in view of Langley and Albrecht's (6) statement that, "The simple α-amino acids did not yield flavianates readily, but did so under unusual conditions" and Crosby and Kirk's (7) failure to secure a crystalline flavianate from glycine. However, glycine flavianate is easily obtained although its separation may be somewhat delayed unless scratching with a glass rod or seeding with a crystal is employed. When 0.75 gm. of glycine was dissolved in 50 cc. of warm water and then 3.5 gm. of flavianic acid added, crystallization promptly followed. After the mixture had stood for 2 days in the refrigerator, the crystals were filtered off and weighed 2.30 gm., equivalent to 60 per cent of the theoretical yield. The recrystallized material began to sinter above 235° and melted at 244-245° with effervescence, giving a deep brownish black residue.

$C_{10}H_6O_8N_2S \cdot C_2H_5O_2N$.	Calculated.	N 10.8,	NH_2N 3.60
	Found.	" 10.8,	" 3.65

The solubility in water was determined at 17° and found to be 52.5 gm. per liter, equivalent to about 10 gm. of glycine per liter. In view of the easy precipitation of glycine by phosphotungstic acid (Sörensen), by silver salts (Kutscher), and by flavianic acid it might well be expected that the presence of much glycine could prove a complicating factor in several of the methods currently used for the estimation of arginine.

In conclusion it may be recorded that hydroxyproline may be synthesized by a reaction similar to that used for the synthesis of γ-hydroxyleucine, epichlorhydrin being substituted for isobutylene oxide, but it is doubtful whether the method has practical advantages over known procedures.

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COCARBOXYLASE HYDROLYSIS BY A WHEAT PHOSPHATASE

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(Received for publication, April 26, 1944)

It was observed that no enzyme addition was required to recover the cocarboxylase as free thiamine in assaying a sample of flour which contained added synthetic cocarboxylase (the pyrophosphoric ester of synthetic thiamine), shown by Weijlard and Tauber (1) to be identical with natural cocarboxylase. This observation indicated that flour contained a phosphatase capable of hydrolyzing cocarboxylase to thiamine. The occurrence of phosphatase in wheat has been demonstrated by Hilbe and Marrow (2) and Sisakyan and Kobyakova (3). This suggested to us that the absence of cocarboxylase in plant materials as observed by Tauber (4), Hennessy and Cerecedo (5), Melnick and Field (6), Booth (7), Shetlar and Lyman (8), and others might be caused by phosphatase activity when the plant materials are extracted for assay.

EXPERIMENTAL

When thiamine is measured by the thiochrome procedure, free thiamine is oxidized by alkaline ferricyanide to thiochrome, which is extracted by isobutanol. Cocarboxylase, however, under these conditions forms an oxidation product which cannot be extracted by isobutanol and thus escapes detection. The difference of solubility of the oxidation products in isobutanol provides a ready means of differentiating thiamine and cocarboxylase. Cocarboxylase is readily converted into thiamine by phosphatase hydrolysis.

A variety of extraction procedures is used to assay flour for thiamine content. The flour may be extracted with potassium chloride solutions (5 to 25 per cent potassium chloride in 0.005 N H_2SO_4) by vigorous shaking. After centrifuging and filtering, an aliquot of the supernatant liquid is used for oxidation of the thiamine to thiochrome. The flour may be extracted with water or buffer solutions and be given an incubation treatment after addition of an enzyme preparation containing phosphatase (such as takadiastase and clarase). After filtration, these extracts may be freed from possible contaminants by the Decalso adsorption procedure and the potassium chloride eluates used for oxidation. The flour may also be taken up in acid solutions which may be heated, cooled, and neutralized before the enzyme preparation is added.

A sample of flour was assayed for thiamine by the potassium chloride extraction procedure described above and was found to contain 0.8 γ per gm. A portion of this flour was enriched by adding crystalline cocarboxylase which when assayed with this procedure was found to contain 3.6 γ per gm. of thiamine. The same value was obtained by the other methods in which an enzyme digestion was used. Quantitative conversion of the cocarboxylase to thiamine was obtained in 1 hour at 27° in the buffer or potassium chloride flour suspensions. Extraction in 0.1 N sulfuric acid, 0.1 N hydrochloric acid, and 2 per cent acetic acid was observed to inactivate the enzyme. Cocarboxylase and thiamine were readily distinguished in this sample by taking up the flour in boiling 0.1 N sulfuric acid and heating to destroy the enzymes at the time of wetting. Whole wheat flour and first and second clear flours, which are comparatively rich in natural thiamine, were also assayed by means of this procedure with the same results as with the enzyme procedures. These results confirm those reported by earlier investigators, which indicate that the natural thiamine of wheat does not occur as cocarboxylase.

TABLE I

Thiamine Assay

Heated				Not heated			
Controls		165 γ cocarboxylase*		Controls		165 γ cocarboxylase*	
Clarase	No clarase	Clarase	No clarase	Clarase	No clarase	Clarase	No clarase
104	97	216	97	98	96	204	203

* The molecular equivalent of 165 γ of cocarboxylase is 112 γ of thiamine hydrochloride.

The efficiency of cocarboxylase hydrolysis by the enzymes in whole wheat flour was compared with those in clarase. Sixteen 25 gm. samples of finely ground whole wheat flour were transferred to 500 ml. volumetric flasks. Eight of these were taken up in 300 ml. of 0.05 M acetate buffer (pH 4.0) at room temperature and to four of them were added 10 ml. of solution containing 165 γ of cocarboxylase, corresponding to 112 γ of thiamine hydrochloride. 1 gm. of clarase (in solution) was added to duplicate samples in each group. After being shaken, the flasks were allowed to stand overnight, then diluted to the mark, and assays for thiamine were made by the Decalso adsorption procedure. The remaining eight samples were taken up in 300 ml. of 0.1 N sulfuric acid and heated 30 minutes in a boiling water bath to destroy the wheat enzymes. The flasks were cooled and adjusted to pH 4.0 with sodium acetate solution before the cocarboxylase-enzyme treatments, as described for the unheated samples, were made. Results of the thiamine assay are shown in Table I.

It is apparent from these data that wheat contains a heat-destructible factor capable of hydrolyzing cocarboxylase. The average thiamine recovery from 165 γ of added cocarboxylase by the clarase and wheat enzymes was 109 γ (theory, 112 γ) or 66 per cent as thiamine chloride hydrochloride equivalent, which is in satisfactory agreement with the 69 per cent equivalent reported by Hennessy and Cerecedo (5).

SUMMARY

Wheat flour has been observed to contain a heat-destructible factor capable of hydrolyzing cocarboxylase. In confirmation of the observations of other investigators, evidence is presented that the natural thiamine of wheat does not occur as cocarboxylase and that an enzyme digestion in the thiochrome assay procedure for wheat flour is not required for the estimation of the natural vitamin.

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A METHOD FOR THE QUANTITATIVE MICRODETERMINATION OF GLUCOSE AND MALTOSE IN MIXTURES*

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(Received for publication, April 14, 1944)

Mixtures of glucose and maltose, which almost always contain dextrins as well, result from the diastatic breakdown of starch or glycogen. In recent years several methods have been developed for the analysis of such mixtures. These have been of two fundamental types, the so called biological ones, which depend upon yeast fermentation under such conditions that only one of the sugars is attacked, and the so called chemical ones, which rely on reagents that will be in some way selective for the different sugars. Examples of the first type are the methods of Somogyi (1) and of Schultz *et al.* (2). An example of the second type is the method of Sichert and Bleyer (3). Somogyi's method seems to be the only one of these that has been used for micro work. Sichert and Bleyer's reagent, which reacts only with glucose in a mixture, unfortunately will not work at all unless there is more than 20 mg. of glucose present.[1]

This paper describes another method, of the chemical type, the possibilities of which seem to have been little explored.[2] This depends on the fact, often overlooked, that, although different reagents (alkaline ferricyanides, copper solutions, or hypoiodites) are reduced by both glucose and maltose, the reducing power of glucose has a different ratio to that of maltose with each reagent. Thus, Braun and Bleyer (5) have pointed out that, with Fehling's solution, maltose and glucose have the same reduction per *mole* of sugar; hence, by *weight*, maltose gives about 53 per cent as much reduction as glucose. The figures of Hanes (6) show that over the first mg. his ferricyanide reagent is reduced nearly as much (about 82 per cent) by maltose as by the same weight of glucose. It should therefore theoretically be possible to determine the reduction of a mixture of the sugars by each of two such reagents, and by the solution of a pair of simultaneous equations to arrive at the separate concentrations of the sugars. For practical reasons, the equations to be solved must be linear and their accuracy must be very high. Furthermore the total reduction of a mixture of glucose and

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* Aided by a grant from Mead Johnson and Company.

[1] An unsuccessful attempt to adapt this reagent to the micro range is reported elsewhere.

[2] The abstract of a paper by Popov has appeared (4). The method outlined there is the same in principle as that described here. Unfortunately, no details are given, and the original paper is not available.

maltose must be exactly equal to the sum of the reductions of the sugars alone.

Reagents—The reagents that were chosen for this investigation were the Hagedorn-Jensen ferricyanide reagent and a modification of Somogyi's "high alkalinity" copper reagent (1). Both of these give results which are highly reproducible, and they show a satisfactory difference in their glucose to maltose reduction ratios.

The Hagedorn-Jensen reagent, as commonly used for the determination of blood sugar, has an upper limit of about 0.3 mg. of glucose. By simply using a larger quantity of the reagent one can extend the range to about 0.8 mg. To 5 cc. of the sugar solution in a 25 × 150 mm. test-tube are added 5 cc. of the Hagedorn-Jensen ferricyanide-carbonate solution. The tube is covered with a bulb blown from glass tubing (6) and heated for 15 minutes in boiling water. After cooling, 5 cc. of the zinc sulfate-potassium iodide solution are added plus 3 cc. of 5 per cent acetic acid. The surplus iodine is titrated with 0.005 N thiosulfate, and the titration volume is subtracted from that given by a blank determination. To avoid errors due to the evaporation of iodine, the glass bulbs are left in place until the instant of titration, and the first few drops of thiosulfate are allowed to wash down the sides of the tube.

Over the working range of this reagent, the deviations from linearity of its reduction (expressed in cc. of thiosulfate solution) by glucose or maltose are less than the experimental error (0.01 cc.). It was not thought practical to try to extend the range any further, since in the case of Hanes' modification (6) there is a definite curvature beyond about 1 mg. The equation for the reagent is $G = 0.1865V$, where G represents mg. of glucose and V is cc. of 0.005 N thiosulfate. The total reduction of a glucose-maltose mixture is equal to the sum of the separate reductions of the sugars, and the reduction of maltose is 0.811 times that of an equal weight of glucose.[3]

The copper reagent gave more trouble. With Somogyi's reagent (1) the reduction of a mixture of glucose and maltose is equal to the sum of the separate reductions, provided both are calculated as glucose. However, the glucose reduction is not linear, whereas over the first mg. the maltose reduction is. The complications introduced by these facts were finally overcome when it was noticed that the glucose-thiosulfate curve straightened out at about 0.4 mg., and was perfectly linear up to values considerably beyond 1.4 mg. The equivalent of 0.4 to 0.6 mg. of glucose was therefore added to the reagent itself. Thus the quantity of glucose actually in the analysis mixture was never less than 0.4 mg., but the reduction due to this first 0.4 mg. occurred also in the blank. When this was subtracted from the total reduction (which happens automatically when the titration value for the determination is subtracted from that for the blank), the difference bore a linear relation to the glucose in the unknown, the line passing through the origin.

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[3] The glucose used for standardization had been recrystallized from acetic acid (7) and dried to constant weight at 78° *in vacuo* over P_2O_5. The maltose was Eimer and Amend's C. P. maltose which was recrystallized from 60 per cent alcohol. Since the amount of water contained in crystalline maltose varies, the quantity of anhydrous sugar was determined in three ways: by acid hydrolysis followed by determination of glucose, by the determination of optical rotation (found, $[\alpha]_D^{20} = +138^\circ$, on the basis of the acid hydrolysis figures), and by drying the sugar at 118° *in vacuo* over P_2O_5. The three methods gave figures concurrent within a few tenths of 1 per cent.

To prepare the reagent, 80 to 100 mg. of glucose are added to each liter of Somogyi's "high alkalinity" reagent, and enough extra potassium iodate (2.5 to 3.5 cc. of 1 N solution) is added to bring the blank up to the usual point. The resulting solution is not perfectly stable; the blank rises by a few hundredths of 1 cc. per day (more rapidly in hot weather), but it need normally not be determined more than once a day. After the blank titration has risen by more than 1 cc., it may be brought back by the addition of more glucose (30 mg. per liter of reagent per cc. of thiosulfate). No variations in the accuracy of the solution have been detected even after a number of such additions. The technique for the use of the reagent is as described by Somogyi (1).

The equation for this modified Somogyi reagent is $G = 0.148V$. The reduction by a given quantity of maltose is 0.578 times that of an equal weight of glucose,[4] and the reductions of glucose and maltose are additive.

Determination and Calculations—Given these reagents, with the characteristics cited, the procedure for the analysis of a mixture of glucose and maltose is as follows: Equal quantities of the mixture are determined, in duplicate, with each of the two reagents. The reduction for each is calculated as glucose. In the following calculations R_{Cu} and R_{Fe} represent these reductions for the copper and ferricyanide reagents respectively. Then from the preceding paragraphs, $R_{\mathrm{Fe}} = G + 0.811M$ (M being the mg. of maltose) and $R_{\mathrm{Cu}} = G + 0.578M$. By subtraction, $R_{\mathrm{Fe}} - R_{\mathrm{Cu}} = 0.233M$, or $M = 4.29(R_{\mathrm{Fe}} - R_{\mathrm{Cu}})$. By substitution of this value of M in either of the first two equations, the value for G is found.

Analysis of Mixtures Containing Reducing Dextrins—Since the reducing power of dextrins is indefinite, no purely chemical method can be used for the complete analysis of mixtures that contain them. The analysis is possible, however, if yeast fermentation is used. If a mixture is fermented with yeast, glucose and maltose are destroyed, and the reduction due to these sugars alone can be measured by the determination of reduction before and after fermentation. In such a case the method of this paper is immediately applicable. A typical experiment of this kind is given in the next section.

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[4] This figure is very different from that employed by Stark (8) who used a factor of about 0.645.

Illustrative Experiments

Glucose and Maltose Alone—Table I shows results obtained with mixtures containing known quantities of glucose and maltose.

Glucose, Maltose, and Dextrins—A sample of dextri-maltose (Mead Johnson and Company) was dried to constant weight *in vacuo* at 78° over P_2O_5. A solution of the dry product was made containing 1.621 mg. in 5 cc. The reduction of 5 cc. samples of this was determined with each of the reagents. Then some of the solution was fermented with 15 per cent of washed fresh yeast for 2.5 hours, no buffer being used. The reduction of the fermented solution was determined. From these data the concentrations of glucose and maltose were calculated. The results are shown in Table II. As a check, 0.1 per cent of dry sodium carbonate was added to some of the dextri-maltose solution, which was then fermented with 10 per cent of washed yeast for 20 minutes (1). The reduction of the fermented solution was determined with the copper reagent. The difference between this re-

TABLE I

Analyses of Mixtures of Glucose and Maltose

Sugars taken		Reduction found, as glucose		Sugars calculated	
Glucose	Maltose	R_{Fe}	R_{Cu}	Glucose	Maltose
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.656	0.163	0.783	0.745	0.651	0.163
0.326	0.491	0.725	0.610	0.325	0.494
0.164	0.163	0.293	0.253	0 154	0.172
0.164	0.653	0.689	0.536	0.157	0.656

TABLE II

Determination of Glucose and Maltose in 1.621 Mg. Samples of Dextri-Maltose, with Use of Fermentation with 15 Per Cent of Yeast

Solutions	Reduction, as glucose	
	R_{Fe}	R_{Cu}
	mg.	*mg.*
Before fermentation..	0.770	0.526
After "	0.261	0.149
Difference (= reduction of glucose + maltose)	0.509	0.377

Sugars calculated, glucose 0.050 mg., maltose 0.566 mg.

duction and that of the original solution should represent glucose, which alone is fermented under these conditions. The maltose can be calculated from the difference in reduction between the solutions which had been fermented with and without sodium carbonate. These results are given in Table III. The agreement between the two methods is satisfactory.[5]

Glucose and Maltose during Fermentation—The following experiment illustrates the potentialities of the method. A solution was prepared containing, in 5 cc., 0.399 mg. of glucose and 0.409 mg. of maltose. To some of this was added 10 per cent of washed yeast, and the mixture was kept at 26–28°. At intervals samples were withdrawn and centrifuged to remove the yeast. Reduction values were determined upon the original solution and also the fermented samples. The results are given in Table IV. It will be noted that all of the glucose was destroyed in the first 15 minutes, whereas some maltose was still present after 2 hours. In Table IV the

TABLE III

Determination of Glucose and Maltose in 1.621 Mg. Samples of Dextri-Maltose, by Fermentation with and without Sodium Carbonate

Solutions	Reduction, as glucose, R_{Cu}
	mg.
Before fermentation	0.526
After buffered fermentation	0.466
" unbuffered fermentation	0.149

Sugars calculated, glucose 0.060 mg., maltose 0.548 mg.

TABLE IV

Changes Due to Fermentation by Yeast in a Mixture Originally Containing 0.399 Mg. of Glucose and 0.409 Mg. of Maltose

Solutions	Reduction		Sugars destroyed	
	R_{Fe}	R_{Cu}	Glucose	Maltose
	mg.	mg.	mg.	mg.
Original solution	0.728	0.626		
After 15 min. fermentation	0.218	0.149	0.395	0.141
" 34 " "	0.157	0.105	0.391	0.222
" 72 " "	0.092	0.062	0.385	0.309
" 120 " "	0.049	0.027	0.401	0.343

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[5] The sodium carbonate buffer was used in this experiment in preference to the phosphate recommended by Stark and Somogyi in a more recent paper (9). It was found that the concentration of phosphate recommended there caused errors of the order of 15 per cent in determinations with the "high alkalinity" reagent.

results were calculated to give the amount of sugar destroyed by the end of the time noted, since this is the only basis on which calculations can be made with the usual fermentation mixture, in which many reducing substances besides glucose and maltose may be present. In the present case, however, the data may also be used to calculate the amounts of sugars still *present* in the solution. If this is done, it will be found that there are errors throughout, which had compensated one another in the first calculations. The figures for the reduction of the original solution will be found to give glucose 0.372 mg. and maltose 0.438 mg.; these are roughly 0.03 mg. too low and too high respectively. The residual glucose after fermentation had begun will be found to be a negative quantity varying from −0.01 to −0.03 mg. Such errors represent about the maximum uncertainty of the method.

DISCUSSION

Any method which depends, as this one does, on the differences between experimental data is susceptible to rather large errors, and these will be larger, the smaller the differences. In the present method the first step in the calculation is a multiplication by a factor of more than 4; the error is thereby increased 4-fold. Since, even with the exceedingly reliable reagents used here, errors of as much as 0.003 mg. are not uncommon, and the errors of the two reagents may be additive, it is clear that an over-all error of 4 × 0.006, or more than 0.02 mg., is to be expected. The method cannot be effective at all unless the most precise technique is used. It is recommended that the glucose factors for the two reagents be checked against each other from time to time, as a new solution, or uncontrolled variations in experimental conditions, may change one of them. All that is required is to determine the reduction of any one solution of pure glucose with both reagents. Assuming that one factor is correct, the other is calculated from it; again it must be stressed that an error of only 0.5 per cent in the relative values of the factors will give an error of 2 per cent in the calculations. Errors affecting both reagents equally (such as variations in the concentration of the thiosulfate solution with temperature) are of slight importance, since such errors are not increased in the calculations. It has been found that, whereas corrections do occasionally have to be made in the glucose-thiosulfate factors, the glucose-maltose ratios for each reagent never vary to a detectable degree.

Such a relatively large error as 0.03 mg. is of course a serious drawback to the method. It can only be said that fermentation methods are susceptible to errors which are as great. There is always dilution of a fermenting solution by the yeast itself: since the sugar must penetrate the yeast cell walls to be fermented, it must be assumed that some of the non-fermentable constituents of the solution will likewise penetrate the yeast, and be removed with it. It has in fact been found in this laboratory that a solution of pure maltose, buffered with sodium carbonate and then treated with yeast, shows an apparent loss of reducing material which can be due only to this dilution effect. The highest theoretical error caused by this with 10 per cent of yeast would of course be 10 per cent (assuming the yeast to be largely water). The errors actually found may be about half this amount. The net error is smaller, in a fermentation experiment, the smaller the reduction of the fermented solution.

SUMMARY

Glucose and maltose have different relative reducing power when determined with different reagents. If the total reduction of a mixture of the two is determined with an alkaline copper and an alkaline ferricyanide reagent, it is possible to calculate the concentration of each in the mixture by the solution of a pair of simultaneous equations. Results obtained in this way are satisfactory, though errors may be as large as 0.03 mg. The causes of error are discussed.

A modification of Somogyi's "high alkalinity" reagent is described, which gives linear proportionality between glucose taken and thiosulfate used over the range 0 to 1 mg.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXVII. THE LIPIDS OF THE HUMAN TUBERCLE BACILLUS H-37 CULTIVATED ON A DEXTROSE-CONTAINING MEDIUM*

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(Received for publication, April 21, 1944)

The high percentage of lipids differentiates the tubercle bacillus and acid-fast bacteria in general from other microorganisms. However, the amount of lipids in acid-fast bacteria varies not only from strain to strain but also with the composition of the medium (1). Results obtained in this laboratory indicate that the lipids of the human tubercle bacillus, Strain H-37, when grown under identical conditions but at different times on the Long synthetic medium (2) show wide variations in the amounts of various lipid fractions and also in chemical constants (3, 4). Similar variations were found in this laboratory in a comparative investigation of five different strains of human tubercle bacilli, four of which had been recently isolated from human cases of tuberculosis (4).

In other investigations[1] conducted in this laboratory on the chemistry of the pathogenic microorganism *Phytomonas tumefaciens* it was found, when the bacteria were cultivated in a synthetic medium in which glycerol was the chief source of carbon, that the growth was slight and the bacterial cells contained only 2 per cent of lipids. However, when sucrose was added to the medium in place of glycerol, the bacterial growth was more luxuriant and the lipid content of the bacteria amounted to about 6 per cent (5).

In view of the results with *Phytomonas tumefaciens* it appeared of interest to study the lipid production and the chemical composition of the lipid fractions of the human tubercle bacillus when cultivated on a modified Long synthetic medium in which glycerol was replaced by dextrose. Through the cooperation of Sharp and Dohme, Glenolden, Pennsylvania, we were provided with a lot of tubercle bacilli, Strain H-37, which had been cultivated on the modified Long synthetic medium. The bacilli were extracted and the lipid fractions were separated essentially as described in previous studies (4).

According to the results of our earlier investigations the alcohol-ether extracts of tubercle bacilli contain phosphatide, acetone-soluble fat, and a wax fraction which melts at about 50°. The phosphatide, as shown by Dr. F. R. Sabin and collaborators (6), possesses important biological properties. In view of the general occurrence of phosphatides in all living cells it is believed that this class of compounds is of vital importance in the life of the cells.

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* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1939–40.

[1] These experiments were conducted in collaboration with The International Cancer Research Foundation, Philadelphia, and Dr. A. J. Riker of the University of Wisconsin.

In the present investigation of the alcohol-ether extract of the bacilli grown on the dextrose-containing medium it was surprising that no phosphatide could be found. The only substances contained in the alcohol-ether extract were acetone-soluble fat and a low melting wax which had the same properties as the wax previously obtained from the mother liquors in the purification of the phosphatides (4). The chloroform-soluble wax isolated from the bacilli after the alcohol-ether extraction also had a low melting point and we were unable to find any high melting wax corresponding to the so called "purified wax" which melts with decomposition at about 205° (7).

The firmly bound lipid isolated after the partly defatted bacilli had been treated with dilute hydrocholoric acid was a wax-like substance with a low melting point.

The results obtained in the present investigation indicate that the composition of the medium on which the bacilli are grown exerts a remarkable influence on the nature of the lipids that are elaborated. The presence of glycerol in the medium is apparently necessary for the production of certain lipid fractions. The importance of this fact must be considered in future investigations.

In view of the differences in lipid components mentioned above it appeared of interest to analyze the acetone-soluble fat and the low melting wax in order to determine whether these fractions contained the characteristic specific constituents previously found in tubercle bacilli grown on the glycerol-containing Long medium. The acetone-soluble fat was found to contain tuberculostearic acid and phthioic acid and the pigment phthiocol, compounds identical with those previously isolated. The fat did not contain any glycerol but a carbohydrate was isolated among the water-soluble constituents. The amount of carbohydrate was too small to permit definite identification but it was probably trehalose.

The low melting wax fractions yielded on saponification a polysaccharide which gave pentose color reactions. The principal ether-soluble component was mycolic acid. There were also found dextrorotatory fatty acids analogous to phthioic acid and also the alcohol phthiocerol.

EXPERIMENTAL

The tubercle bacilli, Strain H-37, were cultivated according to the standard procedure in 1 liter Pyrex bottles for a period of 8 weeks. The medium contained the same ingredients as the Long synthetic medium (2) except that the glycerol was replaced by 10 per cent of dextrose. The cultures, 77 in number, were filtered off and washed with water, after which the bacterial cells were suspended in 3.5 liters of alcohol and shipped to the Sterling Chemistry Laboratory.

To the bacterial suspensions were added 2 liters of alcohol[2] and the mixture was shaken thoroughly. The cells settled rather quickly and after the mixture had stood overnight the clear supernatant was siphoned off. The cells were further dehydrated by treatment with 2 liters of alcohol and the supernatant was siphoned off after the cells had settled. The cells were next extracted four times with 2 liters of alcohol-ether, equal parts, for each extraction. The bacterial cells were then filtered off, washed with alcohol-ether, and extracted with chloroform as will be described later. The alcoholic and alcohol-ether extracts were combined and concentrated under reduced pressure until the ether and most of the alcohol had been removed. The lipids that remained in the aqueous suspension were extracted with ether and the ethereal solution was concentrated to a volume of about 1 liter. The solution was forced through a Chamberland filter under carbon dioxide pressure and the filter was washed thoroughly with ether. The clear filtrate was concentrated to a volume of about 400 cc. and mixed with 500 cc. of acetone. The precipitate which should consist of crude phosphatide was filtered off and washed with acetone.

The precipitate was reprecipitated ten times from ether solution with acetone and was finally obtained as a white powder which weighed 1.5265 gm. The substance melted at 50–56°. Analysis, found, P 0.58, N 0.28 per cent.

The properties of this fraction resemble those of the low melting wax that we have previously found in the mother liquors from the purification of the phosphatide (4). Attempts to isolate a substance from this material corresponding in properties to the usual phosphatide were unsuccessful. The substance was separated into several fractions by treatment with ethyl acetate and precipitations from ether solution with acetone but all the fractions had low melting points and the phosphorus content was low. It is evident therefore that a substance corresponding in properties to the usual tubercle bacillus phosphatide was not present in this lot of bacilli.

Isolation of a Low Melting Wax from the Mother Liquors—The ether-acetone mother liquors resulting from the above operations were concentrated to a volume of about 300 cc. and cooled in ice water, whereupon a white precipitate separated. The precipitate was filtered off, washed with acetone, and dried. The filtrate was concentrated to about 150 cc. and again cooled in ice water. The slight precipitate that separated was filtered off, washed with acetone, and dried. The two precipitates mentioned above were combined, giving a total weight of 8.0763 gm. The material was a nearly white powder which melted at 41–44°. It contained a trace of phosphorus.

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[2] All solvents had been carefully purified and redistilled before they were used. The alcohol had been distilled over potassium hydroxide. The ether was freed from peroxides, dried over calcium chloride, and distilled over potassium hydroxide.

The Acetone-Soluble Fat—The acetone mother liquor from the low melting wax was evaporated to dryness and the residue consisting of acetone-soluble fat was further dried *in vacuo*. It formed a soft brown salve-like mass with a perfume-like odor and it weighed 7.4733 gm.

The Chloroform-Soluble Wax—The bacterial residue from the alcohol-ether extraction was extracted four times with chloroform-ether, equal parts. 2 liters of solvent were used for each extraction. The bacterial cells were filtered off and washed with chloroform-ether and dried. The dry bacterial residue weighed 86 gm.

The chloroform-ether extracts were combined and evaporated to dryness *in vacuo*. The residue was dissolved in ligroin and the solution was forced through a Chamberland filter under carbon dioxide pressure. The filter was washed three times with ligroin. No unfiltrable lipid was noticed on the filter.

The clear filtrate on concentration to dryness left a nearly white waxy residue which weighed 7.8 gm. For purification the substance was dissolved in 100 cc. of ether and precipitated by the addition of 250 cc. of cold acetone. The substance was filtered off and reprecipitated in the same manner. The final purification was carried out by dissolving the precipitate in 125 cc. of ether and adding 250 cc. of cold methyl alcohol. The precipitate was filtered off, washed with methyl alcohol, and dried *in vacuo*. The product was a white powder which weighed 7.1 gm. It melted at about 53° and contained a trace of phosphorus and 0.39 per cent of nitrogen.

The Firmly Bound Lipids—The bacterial residue was examined for firmly bound lipids as follows: 10 gm. of the dried cells were treated with 100 cc. of a mixture of alcohol and ether, equal parts, containing 1.25 cc. of concentrated hydrochloric acid at 45–50° for 2 hours. After the mixture had cooled, the cells were filtered off and washed with alcohol. The cell residue was next extracted under a reflux with 100 cc. of chloroform-ether, equal parts, at 45–50° for 2 hours. After the mixture had cooled, the cells were filtered off and washed with chloroform-ether. The extractions were repeated three times. After the cell residue had been dried, it weighed 8.5164 gm.

The lipids recovered from the acid alcohol-ether extract were a yellowish solid wax-like mass which weighed 0.2127 gm. This fraction was not further examined.

The chloroform-ether extracts were combined and concentrated under reduced pressure to a volume of about 150 cc. To the solution was then added 1.0 gm. of solid sodium bicarbonate in order to remove any hydrochloric acid. After the mixture had stood overnight, the solution was filtered and the filtrate was evaporated to dryness. The residue which weighed 0.8903 gm. was dissolved in 10 cc. of ether and precipitated by addition of 30 cc. of cold acetone. The precipitate was filtered off, washed with acetone, and dried. The substance was a white powder which weighed 0.8392 gm. and it melted at about 44°. It was free from phosphorus.

TABLE I

Lipid Fractions from Tubercle Bacilli, Strain H-37, Grown on Dextrose-Containing Medium

Fraction No.	Description	M p.	N	P	Weight
		°C.	*per cent*	*per cent*	*gm.*
I	Low melting wax	50–56	0.26	0.58	1.5265
II	" " "	41–44		Trace	8.0763
III	Acetone-soluble fat				7.4733
IV	$CHCl_3$-soluble wax	53	0.39	"	7.8000
V	Bound lipids	44		None	9.4858*
Total lipids					34.3619

* Calculated value.

TABLE II

Products Obtained from 77 Cultures of Tubercle Bacilli Cultivated on Dextrose-Containing Medium

	gm.
Total lipids	34.3619
Polysaccharide	1.0887
Extracted bacterial residue	76.5142*
Total dry bacterial mass	111.9648

* Calculated value.

The total bound lipids from 10 gm. of partly defatted bacilli amounted to 1.103 gm., corresponding to 11.03 per cent of the bacterial residue.

The lipid fractions isolated are summarized in Table I.

Isolation of Polysaccharide—The polysaccharide contained in the aqueous solution which remained after the lipids had been extracted from the concentrated alcoholic and alcohol-ether extracts as previously mentioned was isolated in the usual manner by means of basic lead acetate and ammonia.

The lead precipitate was decomposed with hydrogen sulfide and after the lead sulfide had been filtered off, the filtrate was concentrated under reduced pressure to a thick syrup which was dehydrated by grinding under absolute alcohol. The substance weighed 1.0887 gm. but it was not further examined.

The total yield of material obtained from the 77 cultures that were provided for this investigation is summarized in Table II.

It will be seen from the data in Table II that the total lipids amounted to 30.6 per cent of the dry bacilli. Although no phosphatide could be found, it is evident that the total lipid content was about the same as that obtained when the bacilli are cultivated on the ordinary glycerol-containing Long medium.

Analysis of the Acetone-Soluble Fat

Constants of the Fat—The acetone-soluble fat had the following constants: iodine No. (Rosenmund-Kuhnhenn method) 55.5, saponification No. 137, acid No. 68, ester No. 69, Reichert-Meissl No. 6.5, Polenské No. 5.7, unsaponifiable matter 7.36 per cent.

Saponification of the Fat—The fat, 5.84 gm., was saponified by refluxing with 100 cc. of 4 per cent alcoholic potassium hydroxide for 5 hours in an atmosphere of nitrogen. The solution was concentrated by distillation to a volume of about 50 cc., diluted with water, and the unsaponifiable matter was extracted with ether. The unsaponifiable matter obtained on evaporation of the ethereal solution was again refluxed for 2 hours with alcoholic potassium hydroxide, after which the unsaponifiable matter was isolated as mentioned above. The alkaline solutions were combined and examined for water-soluble components and fatty acids.

The unsaponifiable matter formed a thick amber-colored mass that weighed 0.4303 gm., corresponding to 7.36 per cent of the fat. The substance had a pleasant odor and the iodine number was 127.8.

Isolation of the Fatty Acids—The alkaline soap solution was acidified with hydrochloric acid and the fatty acids were extracted with ether. In order to remove phthiocol and certain acids the ethereal solution was extracted with five portions of 1 per cent aqueous sodium bicarbonate solution. The ethereal solution was then dried over sodium sulfate, filtered, and the ether was distilled off. The residue consisting of higher fatty acids weighed 4.06 gm., corresponding to 69.5 per cent of the fat.

Determination of Phthiocol—The sodium bicarbonate extract which was dark red in color was acidified with hydrochloric acid and extracted with ether. The ethereal solution was dried over sodium sulfate, filtered, and the ether was distilled off. The dark colored residue weighed 0.57 gm. The phthiocol contained in this material was isolated as described previously (4) but the amount was too small to be obtained in pure crystalline form. The phthiocol was therefore estimated by the colorimetric method (8) and amounted to about 2.5 mg. The substance showed the reactions of phthiocol. It was easily soluble in dilute sodium bicarbonate, with a bright red color and on acidification the color turned yellowish. A solution in dilute methyl alcohol deposited a few small yellow crystals.

Examination of the Aqueous Solution—The acidified aqueous solution from which the fatty acids had been extracted was examined for trehalose and glycerol by the method formerly described (4). A carbohydrate fraction which gave a Molisch reaction was isolated by means of basic lead acetate and ammonia. The carbohydrate weighed 63 mg. An attempt was made to acetylate this product in pyridine with acetic anhydride and a water-insoluble acetyl derivative was obtained but the amount was too small to permit of its isolation in crystalline form.

The filtrate from the basic lead acetate precipitate was examined for glycerol but none was found.

Examination of the Fatty Acids—The fatty acids, 4.06 gm., were separated by means of the lead salt-ether procedure and gave 0.765 gm. of solid acids and 3.278 gm. of liquid fatty acids. The solid acids were not further examined. The liquid fatty acids were esterified and gave 3.315 gm. of methyl esters which were distilled in a high vacuum at a temperature between 140–210° through a modified Widmer column. The distillate was a yellow oil which weighed 2.093 gm. and the iodine number was 21. The residue in the distilling flask was a thick dark oil, 1.20 gm., and the iodine number was 46. This fraction was not further examined.

The distilled esters after reduction with hydrogen in the presence of platinum oxide were completely saturated, as the iodine number was 0. The hydrogenated esters were saponified with alcoholic potassium hydroxide. The free acids were isolated, after which the lead salt-ether separation was repeated. The solid reduced acid weighed 0.281 gm. and was not further examined. The liquid saturated acids isolated from the ether-soluble lead salt weighed 1.788 gm.; $[\alpha]_D = +4.57°$; mol. wt. 362.

The liquid saturated fatty acids were esterified with diazomethane and were fractionated and refractionated through a modified Widmer column into four fractions. Fractions I and II, total weight 0.63 gm., were optically inactive but the free acids, 0.60 gm., obtained on saponification of the esters were partly solid. The lead salt-ether separation was therefore repeated. The solid acid obtained from the ether-insoluble lead salt weighed 98 mg. The liquid acid isolated from the ether-soluble lead salt weighed 0.497 gm. This acid was a liquid at room temperature. It was optically inactive and the molecular weight determined by titration was 308. This acid would correspond therefore to somewhat impure tuberculostearic acid.

Fraction III of the esters had $[\alpha]_D = +7.7°$ and the free acid, 0.73 gm., obtained on saponification was a thick oil with a neutral equivalent of 396.

Fraction IV of the esters had $[\alpha]_D = +6.5°$. The free acid, 0.286 gm., obtained on saponification was a thick oil and had a neutral equivalent of 436.

No attempt was made to purify these acids further but the properties and dextrorotation would indicate the presence of phthioic acid.

Examination of the Wax Fractions—The wax fractions Nos. I, II, and IV, Table I, had similar properties and were combined. For saponification 15.5 gm. of this material were dissolved in 120 cc. of benzene and 2.5 gm. of potassium hydroxide dissolved in 20 cc. of methyl alcohol were added. The solution turned cloudy and after a short time it gelatinized. After the mixture had stood overnight, it was heated to boiling, whereupon most of the precipitate dissolved but a hard insoluble mass remained on the bottom of the flask. The yellowish supernatant solution was decanted and the flask was rinsed several times with hot benzene.

The benzene-insoluble material was examined for polysaccharide as will be described later.

To the benzene solution were added 2 gm. of potassium hydroxide dissolved in 3 cc. of water and 30 cc. of methyl alcohol and the solution was refluxed in a water bath for about 7 hours. The solution was concentrated by distillation to a volume of about 50 cc. and transferred to a separatory funnel with ether, after which it was shaken with an excess of dilute hydrochloric acid in order to remove the potassium salts. The solution after it had been washed with water until the washings were neutral to litmus was dried over sodium sulfate, filtered, and concentrated to dryness under reduced pressure. The residue was a yellowish oil when warm and a solid wax-like mass at room temperature. The product was dissolved in 50 cc. of ether and diluted with 100 cc. of acetone. On cooling in ice water, a white precipitate separated which was filtered off and washed with acetone. The substance was designated Fraction I. It weighed 6.8 gm.

The filtrate was concentrated to a small volume and diluted with 50 cc. of alcohol. The white precipitate that separated was filtered off, washed with alcohol, and dried. This material, Fraction II, weighed 1.8 gm.

The filtrate from Fraction II was neutralized with alcoholic potassium hydroxide and an alcoholic solution of lead acetate was added in excess. The lead salt that separated was filtered off, washed with alcohol, and dried, thus giving Fraction III.

The filtrate from above was concentrated under reduced pressure to dryness, after which it was transferred with ether and water to a separatory funnel. The aqueous layer was acidified with acetic acid and the mixture was thoroughly shaken, after which the aqueous portion was drawn off and discarded. The ethereal solution after it had been washed free of acetic acid with water was extracted with 2.5 per cent aqueous potassium hydroxide in order to remove any fatty acids that had not been precipitated as lead salts. The alkaline extract yielded Fraction IV.

The ethereal solution was next washed with water, dried over sodium sulfate, filtered, and the ether was distilled off. The residue consisting of neutral material was designated Fraction V.

Fraction I. Crude Mycolic Acid—The substance was dissolved in 50 cc. of ether and the solution was diluted with acetone. On cooling in ice water, a fine globular precipitate separated which was filtered off, washed with acetone, and dried in air. The substance weighed 6.2 gm. and melted at 55–56°. Its properties were similar to those of mycolic acid. The molecular weight determined by titration was 1445. In chloroform solution the substance showed no optical rotation.

Fraction II was probably mainly crude mycolic acid but it was not examined.

Fraction III—The dried lead salt was treated with ether and the insoluble portion was filtered off and discarded. The ethereal solution was freed from lead by means of dilute hydrochloric acid, after which it was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was a slightly yellowish thick oil at room temperature which weighed 1.4 gm.

Titration—0.3372 gm. of acid dissolved in 30 cc. of ether plus 10 cc. of alcohol required 7.94 cc. of 0.1 N KOH. Found, mol. wt. 424.

Rotation—0.5321 gm. of acid dissolved in chloroform and diluted to 10 cc. gave in a 1 dm. tube $\alpha = +0.44°$; hence, $[\alpha]_D = +8.2°$.

Judging by the rotation and molecular weight the acid is somewhat impure phthioic acid.

Fraction IV—The alkaline solution was acidified with dilute hydrochloric acid and extracted with ether. The ethereal solution was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was a thick yellowish oil which partly solidified at room temperature.

Rotation—0.3616 gm. of acid dissolved in chloroform and diluted to 10 cc. gave in a 1 dm. tube $\alpha = +0.20°$; hence $[\alpha]_D = +5.5°$.

Judging by the rotation and properties this acid also contained phthioic acid.

Fraction V—The neutral fraction weighed 0.3 gm. It was a slightly yellowish solid which showed crystalline structure. The substance crystallized from ethyl acetate in aggregates of small prismatic crystals. After two recrystallizations from ethyl acetate 50 mg. of colorless crystals were obtained. The crystals melted at 73–74°. In crystal form and melting point the substance was identical with phthiocerol.

The Polysaccharide—The insoluble product which separated from the alkaline benzene solution of the wax weighed 1.6 gm. It was treated with a small amount of water and gave a cloudy strongly alkaline solution. The solution was slightly acidified with acetic acid, which caused a precipitation of fatty acid. The addition of neutral lead acetate gave a further precipitate which was filtered off and washed with water. The polysaccharide contained in the filtrate was isolated in the usual manner by means of basic lead acetate and ammonia. The lead salt was decomposed with hydrogen sulfide and the filtrate from the lead sulfide was concentrated to a thick syrup which was dehydrated by grinding under absolute alcohol until a white powder was obtained. After the powder had been filtered off, washed with absolute alcohol, and dried *in vacuo*, it weighed 0.5 gm. The substance gave the usual color reactions for pentose with phloroglucinol and orcinol. This polysaccharide is therefore similar to the polysaccharide contained in the wax of the tubercle bacillus cultivated on glycerol-containing medium.

SUMMARY

1. The total lipids of the human tubercle bacillus, Strain H-37, cultivated on a modified Long synthetic medium in which dextrose replaced glycerol, amounted to 30.6 per cent, which is comparable to the lipids obtained from tubercle bacilli grown on the regular Long medium.

2. The lipids did not contain any phosphatide.

3. The only lipid fractions that could be isolated were acetone-soluble fat and a low melting wax.

4. The acetone-soluble fat on analysis was similar in composition to the fat elaborated on a glycerol-containing medium. Tuberculostearic acid and phthioic acid were present and also the pigment phthiocol. The fat contained no glycerol but a carbohydrate which could not be definitely identified.

5. The wax fractions differed from the waxes isolated from the tubercle bacillus cultivated on a glycerol-containing medium in that no high melting wax could be found.

6. The low melting wax, however, gave on analysis certain of the characteristic components of the tubercle bacillus wax; namely, mycolic acid, the alcohol phthiocerol, dextrorotatory fatty acids analogous to phthioic acid, and a polysaccharide that contained pentose.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXVIII. THE LIPIDS OF CELL RESIDUES FROM THE PREPARATION OF TUBERCULIN*

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(Received for publication, April 21, 1944)

In 1939 considerable quantities of tubercle bacilli residues from the preparation of a special lot of PPD (1) under direction of Dr. F. B. Seibert were furnished us through the generous cooperation of Sharp and Dohme, Glenolden, Pennsylvania. The lipid constituents from these cell residues were extracted with two objects in view: (*a*) to obtain larger quantities of certain of the specific lipids, especially tuberculostearic acid, phthioic acid, phosphatide, etc., (*b*) to study the chemical composition of the lipid fractions in comparison with the lipids isolated from the human tubercle bacillus Strain H-37 which have been under investigation in this laboratory during the past several years.

The results of the isolation procedures indicated that the cell residues yielded phosphatide, acetone-soluble fat, and wax that were in many respects similar in solubility properties to analogous fractions previously isolated from Strain H-37. However, in the chemical analysis of the phosphatide prepared from the cell residues decided differences in apparent chemical structure of the polysaccharide component were noted (2). The reason for this difference might depend upon the strain of bacilli or on the treatment of the cultures during the preparation of PPD. In the analyses of the chloroform-soluble wax which will be described in a later report several higher fatty acids were found which were not encountered in the wax obtained from Strain H-37.

In the preparation of PPD the bacterial cultures were heated for 3 hours in an Arnold sterilizer at a temperature of about 100°. The only changes that might occur during the heating would be in the nature of hydrolysis but this operation could not result in the formation of new fatty acids. It would appear therefore that the differences noted in composition of the lipid fractions must depend upon the strain of bacilli that was used.

The only information obtainable concerning the strain of bacilli that was used in the production of this special lot of PPD was that it was the same strain that had been used for many years in the Bureau of Animal Industry, United States Department of Agriculture, for the preparation of tuberculin. When a sample of this strain was transferred from the Biochemic Division, Bureau of Animal Industry, United States Department of Agriculture, to Sharp and Dohme, April 17, 1933, it was designated "O. T. strain." In the Sharp and Dohme laboratory it was labelled "strain No. 2067." It must be stated that the origin of this strain cannot be determined at this time.

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* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1939–40.

It is evident from the results obtained that Strain 2067 produces phosphatide and wax that differ to some extent in composition and cleavage products from those found previously in Strain H-37, although both strains had been cultivated under identical conditions on the Long synthetic medium (3).

EXPERIMENTAL

The tubercle bacilli had been cultivated for a period of 8 weeks on the Long synthetic medium (3). The cultures were then heated in an Arnold sterilizer for 3 hours. After the material had cooled, the bacterial cells were filtered off, washed with distilled water, and suspended in an adequate quantity of denatured alcohol contained in 5 gallon Pyrex bottles and shipped to the Sterling Chemistry Laboratory. Six such bottles were received in good condition.

To each bottle were added 5 liters of alcohol[1] and the contents were thoroughly mixed. The bottles were allowed to stand at room temperature for a couple of days until the bacterial cells had settled, after which the supernatant solution was siphoned off. To the cell residue were added another 5 liters of alcohol with thorough mixing and after the cells had settled the clear supernatant was siphoned off. The cells were next extracted four times at room temperature. 6 liters of alcohol and ether, equal parts, were used for each extraction. After the cells had settled, the supernatant was siphoned off but after the last extraction the cells were filtered off and washed with alcohol-ether.

The cell residue was next extracted exhaustively with a mixture of chloroform and ether, equal parts, for the removal of chloroform-soluble wax. Following each extraction the cells were filtered off and washed with chloroform-ether.

For the removal of firmly bound lipids, the cell residues, after the chloroform-ether extraction, were treated with a mixture of 2 liters of chloroform and 2 liters of ether containing 50 cc. of concentrated hydrochloric acid. Following this treatment the cells were further extracted with chloroform-ether, equal parts. The cells were removed by filtration and washed with chloroform-ether.

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[1] All solvents had been carefully purified and redistilled before they were used. The alcohol had been distilled over potassium hydroxide. The ether was freed from peroxides, dried over calcium chloride, and distilled over potassium hydroxide.

Separation of the Lipid Fractions—The alcohol and alcohol-ether extracts were combined and concentrated under reduced pressure until the lipids began to separate due to the presence of water derived from the bacterial cells. The lipids were extracted with several portions of ether, and the ethereal extracts were forced through Chamberland filters under carbon dioxide pressure. The lipids were separated into phosphatide, a low melting wax, and acetone-soluble fat by the method described in a former report (4).

The chloroform-ether extracts were concentrated at a temperature of about 40°, a current of carbon dioxide being passed through the solution, until the ether was removed. The chloroform solution was then forced through a Chamberland filter under carbon dioxide pressure. In only one case was a small amount of unfiltrable lipid (5) observed. The clear filtrate was concentrated under reduced pressure to dryness.

The Firmly Bound Lipids—The combined chloroform-ether extracts were freed of hydrochloric acid by shaking with solid sodium bicarbonate. After filtration the extracts were concentrated to a small volume and forced through a Chamberland filter under carbon dioxide pressure. A very small amount of unfiltrable bound lipids was obtained. For purification the bound lipids were precipitated from ether solution by addition of acetone and cooling in ice water until a nearly white powder was obtained. The mother liquors on evaporation to dryness yielded a soft wax-like residue. The firmly bound lipids weighed 115.7 gm., of which 6.16 gm. were unfiltrable lipids.

Isolation of Polysaccharide—After the alcohol and alcohol-ether extracts of the bacterial residues had been concentrated and the lipids contained therein had been extracted with ether, there remained an aqueous solution derived from the moist bacteria. This solution was concentrated under reduced pressure to a volume of about 400 cc. and a solution of neutral lead acetate was added in slight excess. The resulting precipitate was filtered off, washed with water, and discarded. The filtrate was concentrated to a volume of about 150 cc. and the polysaccharide was precipitated by means of basic lead acetate and ammonia. The lead salt was filtered off, washed with dilute ammonia, suspended in water, and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was concentrated to a thick syrup and the latter was triturated under absolute alcohol until a nearly white powder was obtained which was filtered off, washed with absolute alcohol, and dried *in vacuo*. The amounts of polysaccharide obtained were small, the total yield being 20.1 gm. The product gave the Molisch reaction and the usual pentose color reactions.

Results

The phosphatides were isolated separately from each lot of bacterial residues by addition of acetone to the ether solution and purified by from ten to fourteen precipitations in the same manner. The final precipitation was done by pouring the ethereal solution into a large volume of ice-cold acetone, whereupon the phosphatide separated as a nearly white compact powder. The analyses of the phosphatide fractions are given in Table I. It will be noted that all the fractions were very similar in composition.

Low Melting Wax Recovered from Mother Liquors in Purification of the Phosphatide—The mother liquors from the purification of the phosphatide were concentrated by distillation until the ether was removed. The solution, on cooling in ice water, deposited a brownish solid precipitate which was filtered off and washed with cold acetone. A total of 234 gm. of material was obtained and it was purified by precipitation from ether solution first by addition of acetone and later by methyl alcohol until a nearly white powder resulted which weighed 163 gm. and melted unsharply at 44–51°. The mother liquors on concentration gave a dark colored, soft solid mass.

TABLE I

Composition of the Phosphatide Preparations

Lot No.	Weight	Phosphorus	Nitrogen	M.p., with decomposition
	gm.	*per cent*	*per cent*	°C.
1	31.77	3.10	0.78	202–215
2	49.64	3.52	0.61	204–205
3	46.60	3.50	0.54	190–195
4	39.24			
5	41.08	3.54	0.55	200–203
6	37.22	3.78	0.51	190–195

The Acetone-Soluble Fat—The mother liquors after the low melting wax was removed as mentioned above were concentrated to dryness. The residue was a reddish brown thick oil that weighed 218.9 gm.

Isolation of Phthiocol and Anisic Acid from the Acetone-Soluble Fat—The acetone-soluble fat was dissolved in ether and extracted with 5 per cent sodium bicarbonate solution until the extracts were colorless. The ethereal solution was washed with water, dried over sodium sulfate, filtered, and the ether was distilled off. The dark colored, oily fat residue weighed 209.8 gm.

The sodium bicarbonate extracts, which were bright red in color, were combined and examined for phthiocol and any acids that might be present by the methods previously described (4). The only substances that could be identified were phthiocol and anisic acid. Estimation of phthiocol in the sodium bicarbonate solution by the colorimetric method (6) indicated that 0.1763 gm. of free phthiocol was present. The substance was isolated and purified by repeated recrystallization from methyl alcohol and gave 57.8 mg. of yellow prismatic needles which melted at 172–173°.

The anisic acid was isolated and purified by crystallization from water. The yield was 0.1818 gm. of colorless needles, m.p. 283–284°.

A number of other acids were present but could not be identified.

A more complete analysis of the acetone-soluble fat will be reported in a separate paper.

The Chloroform-Soluble Wax—The crude chloroform-soluble wax obtained on evaporation of the chloroform solution as mentioned earlier weighed 308.6 gm. For purification the wax was dissolved in ether and precipitated by the addition of methyl alcohol and cooling in ice water. These operations were repeated until a nearly white powder was obtained. The purified wax weighed 232.6 gm. and melted at 53°.

The mother liquors on evaporation to dryness gave a soft salve-like mass which weighed 75.5 gm.

The properties and composition of the purified wax will be reported in a separate paper.

SUMMARY

The lipids contained in tubercle bacilli residues from the preparation of PPD have been extracted and separated into phosphatide, low melting wax, acetone-soluble fat, chloroform-soluble wax, and firmly bound lipids.

In solubility these lipids resemble analogous fractions previously isolated from tubercle bacilli, Strain H-37, but certain differences have been observed in chemical composition. The differences in composition of the lipids must be due to the variety of the strain of bacilli grown for the production of PPD.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXIX. THE COMPOSITION OF THE ACETONE-SOLUBLE FAT OF CELL RESIDUES FROM THE PREPARATION OF TUBERCULIN*

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(Received for publication, April 28, 1944)

As described in the preceding paper (1) a quantity of acetone-soluble fat had been prepared from tubercle bacilli residues which remained from the production of a special lot of tuberculin or PPD. This fat has been examined in order to determine whether it contained characteristic compounds such as tuberculostearic acid (2, 3), phthioic acid (2, 4), the pigment phthiocol (5), and other constituents similar to those previously isolated from the acetone-soluble fat of the human tubercle bacillus, Strain H-37.

It has been shown in a previous report (6) that the carbohydrate fraction contained in the phosphatide isolated from the cell residues from the preparation of PPD differed from the carbohydrate contained in the phosphatide prepared from living tubercle bacilli, Strain H-37. It appeared of interest therefore to determine whether the acetone-soluble fat also differed in composition.

After saponification of the fat and extraction of the fatty acids the water-soluble component was isolated and identified as trehalose, thus confirming our earlier observations that trehalose occurs in the fat of the tubercle bacillus in place of glycerol (7). Phthiocol and anisic acid were also isolated and identified.

The fatty acids were separated into solid and liquid acids by the lead salt-ether procedure. The unsaturated acids present in the liquid acid fraction were hydrogenated and removed as ether-insoluble lead salts. The original solid acids and the solid acids obtained on hydrogenation were not further investigated.

The saturated liquid fatty acids obtained from the ether-soluble lead salts were converted into methyl esters and the esters were separated by fractional distillation. The lower boiling ester fraction was optically inactive and on saponification it gave an optically inactive acid which was liquid at room temperature. In general properties this acid corresponded to tuberculostearic acid. This fraction was, however, not homogeneous since, when its methyl ester was subjected to careful fractionation, it was separated into several fractions and yielded different acids on saponification. One of these acids appeared to be a new acid which showed a slight levorotation and corresponded to the formula $C_{19}H_{38}O_2$. About one-third of the original ester was apparently pure methyl tuberculostearate. The end-fractions were contaminated with dextrorotatory esters.

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* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1941–42.

‡ Holder of a National Tuberculosis Association Fellowship at Yale University, 1939–40.

The higher boiling ester fraction was dextrorotatory and on saponification it gave a dextrorotatory acid which in its general properties resembled phthioic acid. A more careful fractionation of this acid, which will be described in a later report, showed that it was a mixture of several higher acids that varied in the magnitude of dextrorotation and in composition, but one fraction corresponded in properties to phthioic acid.

The results obtained in the present investigation indicate that the fat contained some of the characteristic compounds previously isolated from the fat obtained from Strain H-37, but in addition different liquid saturated fatty acids were also present.

EXPERIMENTAL

The isolation of the acetone-soluble fat was described in the preceding paper (1). The following constants were determined: saponification No. 213.3, iodine No. 82.3, Polenské No. 0.52, Reichert-Meissl No. 3.45, free fatty acids 25.14 per cent, neutral fat 69.38 per cent.

Saponification of the Fat—Of the original fat, which weighed 209.8 gm., 5.2 gm. were reserved. To the remainder were added 9.1 gm. of a mixture of free fatty acids and neutral fat recovered from analytical operations; hence a total of 213.7 gm. of fat was saponified by refluxing for 6 hours with an excess of 5 per cent alcoholic potassium hydroxide in an atmosphere of nitrogen. The solution after it had been concentrated to about one-half of its original volume was diluted with water and the unsaponifiable matter was extracted with seven portions of ether. The ethereal solution was evaporated to dryness and the residue was refluxed for 6 hours with alcoholic potassium hydroxide. After the saponification mixture had been concentrated, it was diluted with water and again extracted with several portions of ether.

The ethereal solution was washed with water, dried over sodium sulfate, filtered, and the ether was removed by distillation. The residue consisting of unsaponifiable matter was a thick, dark colored viscous mass that weighed 39.15 gm., corresponding to 18.3 per cent of the fat. The unsaponifiable matter was highly unsaturated and had an iodine number of 224.

Isolation of the Fatty Acids—The combined alkaline solutions after the unsaponifiable matter had been removed were acidified with hydrochloric acid and the fatty acids were extracted with ether. The aqueous solution was examined for trehalose as will be described below.

The ethereal solution containing the fatty acids was first extracted with several portions of 0.5 per cent aqueous sodium bicarbonate in order to remove phthiocol, anisic acid, and other substances. The separation of these products will be described later. The ethereal solution was next washed with water, dried over sodium sulfate, filtered, and the ether was distilled off. The crude fatty acids after drying *in vacuo* weighed 142.85 gm., corresponding to 67 per cent of the original fat.

Examination of the Sodium Bicarbonate Extract—The solution was acidified with hydrochloric acid and extracted with ether. The ethereal extract was washed with water, dried over sodium sulfate, filtered, and the ether was distilled off. The residue after drying *in vacuo* weighed 10.32 gm. By means of the methods described earlier (5) it was possible to isolate phthiocol and anisic acid from this fraction. The balance of the material represented a mixture of acids from which no pure substance could be isolated.

The crude phthiocol fraction weighed 0.730 gm. For purification the substance was sublimed. The sublimate consisted of delicate yellow prisms and weighed 162 mg., m.p. 174°.

The crude anisic acid weighed 0.645 gm. It was purified by several recrystallizations from dilute alcohol and was obtained as delicate colorless prismatic needles which melted at 183–184°.

Isolation of Trehalose—The acidified aqueous solution from which the fatty acids had been extracted was neutralized with potassium hydroxide and evaporated to dryness under reduced pressure. The residue was extracted with 70 per cent alcohol for the removal of water-soluble components. The carbohydrate contained in the solution was isolated as described in a previous paper (8). The crude carbohydrate in the form of a white powder weighed 4.28 gm. The substance was acetylated by refluxing with acetic anhydride and fused sodium acetate. The reaction mixture was poured into water and shaken until the acetic anhydride was decomposed, after which the acetyl derivative was extracted with chloroform. The chloroform solution was washed with water until the washings were neutral to litmus, dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was recrystallized twice from methyl alcohol. Colorless needle-shaped crystals were obtained which weighed 3.50 gm. The crystals melted at 78–80°; $[\alpha]_D$ in $CHCl_3 = +162.9°$. The crystal form and properties show that the substance was trehalose octaacetate.

Separation of the Fatty Acids—The total fatty acids, 142.8 gm., were separated into solid and liquid acids by the usual lead salt-ether procedure. The regenerated solid and liquid acids were subjected a second time to the lead salt-ether separation.

The Solid Acids—The solid fatty acids isolated from the ether-insoluble lead salts weighed 36.29 gm. and were nearly free from unsaturated acids since the iodine number was only 2.9. The only acid identified in this mixture was hexacosanoic acid. The other components appeared to be a mixture of palmitic and stearic acids.

Isolation of Hexacosanoic Acid—The solid fatty acids were dissolved in 300 cc. of hot alcohol. As the solution cooled, thin plate-shaped crystals separated. The crystals, after they had been filtered off, washed with alcohol, and dried, weighed 1.85 gm. and melted at 82–84°. The acid after it had been recrystallized from alcohol and from acetone melted at 86° and there was no depression of the melting point when mixed with normal hexacosanoic acid which melted at 88°. The molecular weight determined by titration was 389. The data indicate that the acid was nearly pure normal hexacosanoic acid.

The Liquid Fatty Acids—The liquid fatty acids isolated from the ether-soluble lead salts formed a dark colored oil which weighed 96.8 gm. and had an iodine number of 68.3. The acids were esterified by refluxing with absolute methyl alcohol containing 5 per cent of dry hydrochloric acid. The ester was isolated and distilled in a high vacuum through a modified Widmer column. The distillate was a light yellow oil which weighed 66.9 gm. and had an iodine number of 50.8. The non-volatile portion of the ester weighed 31.7 gm. and had an iodine number of 117. This fraction was not further examined.

The distilled ester was dissolved in a mixture of alcohol and ether and reduced with hydrogen in the presence of platinum oxide. The reduced ester was completely saturated, as it absorbed no iodine. It was saponified and the free acids were isolated. In order to remove the solid reduced acids the lead salt-ether separation was repeated twice.

The liquid saturated acids obtained from the ether-soluble lead salts weighed 44.17 gm. and the molecular weight by titration was 362.8. At room temperature it was a thick oil.

The solid reduced acids obtained from the ether-insoluble lead salts weighed 17.87 gm. This fraction was not further examined.

The Liquid Saturated Fatty Acids—The liquid saturated fatty acids were esterified by refluxing with absolute methyl alcohol containing 5 per cent of dry hydrochloric acid and the ester was isolated in the usual manner. The ester which presumably consisted of a mixture of methyl tuberculostearate and phthioate was fractionated at a pressure of 0.001 mm. through a modified Widmer column. On redistillation at the same pressure eight fractions were collected.

The first two fractions having a total weight of 17.4 gm. were distilled with the oil bath at a temperature of 160–205°. These fractions were optically inactive and corresponded in general properties to the methyl ester of tuberculostearic acid.

The next intermediate fraction weighed 2.02 gm. and showed a dextrorotation of 0.55°.

The next five fractions were distilled with the temperature of the oil bath from 234–300°. The total weight of these fractions was 25.19 gm. and they varied in specific rotation from +10.3° to +11.9°. The dextrorotation would indicate that these fractions consisted largely of methyl phthioate. However, the molecular weights of these fractions calculated from the saponification equivalents varied from 425 to 430. Since methyl phthioate has a molecular weight of 410, the values found would indicate that the ester was not pure.

It will be shown in a subsequent paper that the dextrorotatory ester fractions described above yielded on careful fractionation a series of esters that varied in optical rotation and in molecular weight.

Examination of Optically Inactive Liquid Saturated Fatty Acid Ester[1]—The optically inactive ester mentioned above was saponified and the free acid was isolated and subjected to a second lead soap-ether treatment. A small amount of ether-insoluble lead salt was removed by centrifugation and discarded. The acid obtained from the ether-soluble lead salt weighed 15.6 gm.

For further purification the acid was esterified with diazomethane and the methyl ester was carefully fractionated through a special column at a pressure of 1.5 mm.[2] Several different ester fractions were separated and examined.

A small amount, 1.23 gm., of esters of normal solid acids was separated during the fractionation. The solid acids thus removed appeared to consist of a mixture of myristic, palmitic, and stearic acids.

The esters of the liquid saturated acids on continued fractionation were separated into three principal fractions as indicated below.

Fraction I—The ester, 0.49 gm., distilled at a column temperature of 163°. The ester showed a low levorotation, $[\alpha]_D^{23}$ in ether = $-1.25°$; n_D^{45} 1.4368. The free acid obtained on saponification of the ester was liquid at room temperature; $[\alpha]_D^{23}$ in ether = $-1.2°$. The molecular weight by titration was 299.

$C_{19}H_{38}O_2$ (298). Calculated, C 76.51, H 12.75; found, C 76.47, H 13.10

The results indicate that this acid is a new levorotatory acid of the formula $C_{19}H_{38}O_2$ and hence isomeric with tuberculostearic acid.

Fraction II—The ester, 3.1 gm., distilled at a column temperature of 170–180°. The index of refraction was n_D^{45} 1.4372. The ester was optically inactive and appeared to be pure methyl tuberculostearate. The free acid was liquid at room temperature but it solidified in ice water. The molecular weight determined by titration was 299. The calculated molecular weight for $C_{19}H_{38}O_2$ is 298.

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[1] We are indebted to Dr. Leonard G. Ginger and Dr. Sidney F. Velick of this laboratory who carried out these experiments.

[2] The fractionations were carried out by Dr. Sidney F. Velick through a special column which he had designed; a description of its construction and resolving power will be published elsewhere.

Fraction III—This material, 3.15 gm., consisted of various end-fractions which had been collected at column temperatures between 180–200°. These fractions showed specific dextrorotations from +0.6° to +3.4° and were therefore contaminated with small amounts of dextrorotating acids.

SUMMARY

The acetone-soluble fat isolated from tubercle bacilli residues from the preparation of tuberculin has been examined for characteristic constituents previously found in the fat of the human tubercle bacillus, Strain H-37.

The fat was similar in composition to the fats previously analyzed and consisted of fatty acid esters of the disaccharide trehalose.

Among the cleavage products liberated on saponification were the pigment phthiocol and anisic acid.

The liquid saturated fatty acids contained in addition to tuberculostearic acid and phthioic acid several other higher liquid saturated fatty acids.

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CHEMICAL STUDIES ON POWDERED KERATINS

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(Received for publication, May 15, 1944)

Numerous studies have been made on the alteration of the characteristic properties of keratins by such chemical agents as alkali, acid, sulfide, sulfite, amines, guanidine, urea, and oxidizing and reducing agents. An increased solubility and a decreased resistance to enzyme action were usually observed. Similar changes have been produced by the mechanical breakdown of wool keratin in a ball mill. As the wool was ground, an increasing fraction of its nitrogen and sulfur became soluble in water (1, 2), and the powdered material was digestible by enzymes *in vitro* (1) and *in vivo* (3). Similar chemical studies have been undertaken to determine the changes that occur when keratins other than wool are ground in the ball mill.

EXPERIMENTAL

The keratins employed in this investigation were human hair, chicken feathers, duck feathers, porcupine quill, turkey quill, and turkey fan. Considerable quantities of each material were carefully cleaned and defatted. After the material was cut to uniform size in a Wiley mill, the keratins were powdered and screened as heretofore described (2). Prior to analysis samples were dried in a vacuum oven at 65° and stored in a vacuum desiccator over phosphorus pentoxide.

Extracts of the powdered material were prepared by suspending samples (6 to 7 gm.) in water (35 ml.) with stirring for 15 minutes. The suspensions were centrifuged, the supernatant liquid was removed, and the process repeated three times. The residue after extraction was dried in a vacuum oven.

Total nitrogen was determined by Kjeldahl and total sulfur by the Benedict-Denis methods. Cystine determinations were made by the methods of Shinohara (4) and Sullivan (5). The inorganic sulfate content of the extracts was determined gravimetrically after precipitation with barium chloride.

Separation of a powdered keratin into fractions of different particle size was deemed unnecessary because little chemical variation was observed between these fractions. This confirms the observations made on powdered wool (2); chemical composition of the particles depended not on particle size but rather on duration of the grinding process. The data presented in Tables I and II are therefore restricted to alterations in the composition of keratins as affected by the length of grinding. The various keratins were usually ground by 500,000 and 1,000,000 revolutions of the ball mill. To obtain more complete information about that keratin, one lot of chicken feathers ground for a total of 1,000,000 revolutions was sampled at intervals of 100,000 revolutions and another portion was ground by 1,500,000 revolutions.

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* The experimental data are taken from a dissertation submitted by Beatrice Edwards to the Faculty of the Graduate College of the State University of Iowa in partial fulfilment of the requirements for the degree of Doctor of Philosophy, July, 1942.

TABLE I

Composition of Keratins

Material	Ball mill revolutions	Nitrogen	Sulfur	Cystine		Ash
				Sullivan	Shinohara	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Hair	None	15.80	5.48	16.45	16.35	0.8
	500,000	15.77	5.54	13.75	13.75	0.9
	1,000,000	15.41	5.59	6.74	9.15	2.7
Turkey fan	None	15.93	2.87	7.65	7.89	1.7
	500,000	15.95	2.82	5.24	5.84	1.9
	1,000,000	15.81	2.93	2.67	2.01	9.1
" quill	None	16.03	2.56	7.80	7.48	0.3
	500,000	15.90	2.71	5.01	5.09	0.5
	1,000,000	16.27	2.63	5.37	5.62	0.6
Duck feathers	None	15.74	3.25	10.50	9.50	1.1
	500,000	16.17	3.13	6.68	7.24	1.7
Porcupine quill	None	15.96	2.01	5.11	6.21	0.2
	500,000	15.96	2.23	4.21	3.73	0.5
Chicken feathers	None	15.95	2.66	5.80	7.00	1.1
	100,000	15.88	2.99	5.26	6.25	1.4
	200,000	15.96	2.97	4.67	6.16	1.5
	300,000	15.96	2.92	4.33	5.42	1.7
	400,000	15.80	2.90	4.05	4.82	
	500,000	15.81	2.90	3.99	4.42	1.5
	600,000	16.16	2.88	3.73	4.16	3.0
	700,000	16.19	2.95	3.66	3.38	
	800,000	16.04	2.94	3.16	2.20	4.6
	900,000	16.45	2.91	2.58	1.88	
	1,000,000	16.03	2.84	1.63	1.96	10.4
	1,500,000	15.86	2.84	1.48	2.43	9.3

The nitrogen content of the keratins was not appreciably altered by the grinding process, but the water-soluble nitrogen increased with the duration of grinding except in the case of turkey quill. The water-soluble nitrogen could be completely precipitated by saturation of the solution with sodium sulfate; 20 per cent trichloroacetic acid precipitated 80 to 95 per cent of it, whereas 20 per cent sulfosalicylic acid precipitated none of it. This suggests that the soluble fragments are of the approximate molecular size of peptones. The residue after extraction contained slightly less nitrogen than the unextracted material.

As with powdered wool, the greatest changes were observed in the cystine content of the keratins. The values by both the Sullivan and Shinohara methods are given in Table I. Since the Shinohara method reacts to metals, and iron from the ball mill was present in many of the hydrolysates, the values given by the Sullivan method are considered more accurate and are used throughout in Table II. Qualitative tests for cystine on water extracts were uniformly negative. After hydrolysis with 20 per cent hydrochloric acid, extracts exhibited cystine values that increased with the duration of grinding. The residues after extraction contained a smaller amount of cystine than the unextracted material, except in the powdered chicken feathers.

TABLE II

Aqueous Extracts of Powdered Keratins

Material	Ball mill revolutions	Nitrogen content of			Cystine content of			Sulfur content of					
									Water-soluble fraction				
		Unextracted (*a*)	Water-soluble fraction	Residue	Unextracted (*b*)	Water-soluble fraction	Residue	Unextracted (*c*)	Total (*d*)	Inorganic sulfate (*e*)	Cystine (*f*)	Partially oxidized (*d* − (*e* + *f*))	Residue
	millions	*per cent*	*per cent of (a)*	*per cent*	*per cent*	*per cent of (b)*	*per cent*	*per cent*	*per cent of (c)*	*per cent of (d)*	*per cent of (d)*	*per cent of (d)*	*per cent*
Hair	0.5	15.80	4.16	15.43	13.75	0.39	12.61	5.54	1.58	72.7	19.6	7.7	5.09
	1.0	15.41	12.72	15.00	6.74	2.36	6.48	5.59	10.10	43.7	17.2	39.1	5.20
Turkey fan	0.5	16.12	13.86	15.52	5.24	1.44	3.83	2.82	12.87	35.5	5.5	59.0	2.80
	1.0	15.81	22.80	15.37	2.67	9.43	2.04	2.93	21.80	24.0	19.3	56.7	2.54
" quill	0.5	15.90	14.87	15.83	5.01	1.43	4.59	2.71	10.30	35.2	6.8	58.0	2.34
	1.0	15.31	11.86	15.94	5.37	3.62	4.25	2.63	8.15	38.9	22.2	38.9	2.57
Duck feathers	0.5	16.17	14.78	15.27	6.68	3.34	5.23	3.13	14.77	42.2	12.6	45.2	3.66
Porcupine quill	0.5	16.22	16.11	15.84	4.21	3.68	3.18	2.23	16.00	49.1	11.8	39.1	2.27
Chicken feathers	0.5	15.91	11.71	15.60	3.99	2.07	4.23	2.90	11.25	56.1	6.7	37.2	2.90
	1.0	16.03	40.70	14.97	1.63	15.06	2.81	2.84	42.80	36.6	5.4	58.0	2.67
	1.5	15.86	42.50	15.17	1.48	34.40	2.60	2.65	39.80	38.9	12.8	48.3	2.78

The total sulfur content of the powdered keratins and of the residues after extraction was affected only slightly by the grinding process. The water-soluble extracts contained increasing amounts of sulfur as grinding was prolonged. In general the sulfur values for these fractions closely paralleled the nitrogen values. Inorganic sulfates accounted for approximately one-fourth to one-half of the total soluble sulfur, while cystine sulfur made up about 5 to 22 per cent of the total. Partially oxidized sulfur of the extracts calculated by difference accounted for approximately 40 to 60 per cent of the soluble sulfur.

With the exception of human hair, the keratins used in this investigation exhibited more pronounced chemical changes than wool when they were ground in a ball mill. A larger fraction of the nitrogen and sulfur of the original keratin became soluble in water and the decrease in the cystine content was usually greater than that observed with wool keratin. These differences were especially apparent in the grinding of chicken feathers. The degradation of these proteins in the ball mill was accompanied by oxidation as noted previously (2); only 5 to 22 per cent of the water-soluble sulfur was in the form of cystine, while the remainder was either partially or completely oxidized to inorganic sulfates.

SUMMARY

1. When the keratins of human hair, turkey feathers, duck feathers, chicken feathers, and porcupine quill were ground in a steel ball mill, a marked decrease in the cystine content of the powdered material was observed. The nitrogen and sulfur content of the keratins was not appreciably affected.

2. Aqueous extracts contained increasing amounts of nitrogen, cystine, inorganic sulfates, and partially oxidized sulfur compounds. With the exception of human hair, a larger fraction of the original keratin was soluble in water than that from wool.

3. Mechanical degradation of these keratins was apparently accompanied by oxidation, as evidenced by the decrease in cystine sulfur and the presence of one-fourth to one-half of the water-soluble sulfur in the form of inorganic sulfates.

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THE PHOTOFLUOROMETRIC DETERMINATION OF ATABRINE

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(Received for publication, May 6, 1944)

Since the introduction of atabrine more than a decade ago, at least a score of papers have appeared dealing with its determination in the biological fluids and tissues. The early work of Hecht (1) and of Tropp and Weise (2) established the general colorimetric methods upon which later contributions (3–9) were based. Gentzkow (10) suggested a new approach; *i.e.*, a nephelometric method involving the use of modified Tanret's reagent. With the help of these methods, the various authors and their coworkers have recorded a valuable body of data having to do with the elimination of atabrine in urine and feces, and its deposition in the various tissues. Neither the colorimetric nor nephelometric approach, however, is delicate enough for the estimation of atabrine in the blood of subjects undergoing normal therapeutic treatment. For this purpose, only the extreme sensitivity of the photofluorometric method suffices.

As early as 1933, Massa (11) published some observations on the fluorescence of extracts prepared from the urine and blood of subjects undergoing atabrine medication. In 1935, Roncoroni (12), of the same laboratory group, extended Massa's observations and stated that, for concentrations less than 1 mg. per cent, the fluorescence intensity of atabrine solutions under Wood's light is proportional to the concentration of the drug.

During 1943, the need for more extensive knowledge concerning the distribution of atabrine in the body partly manifested itself in the publication of several papers. The data of Dearborn, Kelsey, Oldham, and Geiling (13) and of Scudi and Hamlin (14) indicate that their methods, while suitable for the estimation of urine, fecal, and tissue concentrations, were not sufficiently sensitive for blood assays. Each of these methods depends upon measuring the fluorescence produced in amyl alcohol solutions of atabrine base. The same solvent was utilized in 1939 by Dubost and Allinne (9) in conjunction with a visual type fluoroscope.

With the publication of the work of Masen (15) and especially of Brodie and Udenfriend (16), the photofluorometric assay of atabrine in blood attained the status of a truly useful method. Each of these is described as a simple method by the authors. Nevertheless, each involves inconvenient complications. The method to be described here is felt to be simpler than any heretofore published. At the same time, no concession has been made as to sensitivity. Indeed, if anything, the sensitivity of the general method has been somewhat improved.

This improvement follows largely from the observation of Ferrari (17) that the fluorescence of solutions of acridine derivatives is intensified by the addition of caffeine sodium benzoate. A survey of the effect of various media on the fluorescence of atabrine led the present authors to adopt Ferrari's reagent. Table I indicates the surprising extent to which choice of solvent determines the fluorescence intensity of atabrine. The figures are not intended to be taken as more accurate than ±10 per cent.

TABLE I

Relative Net Fluorescence of 0.5 γ of Atabrine in 16 Ml. of Various Solvents ($H_2O = 100$)

Solvent No.	Fluorescence
1. 0.1 N HCl	143
2. 0.1 " NaOH	343
3. 1% borax	343
4. 10 ml. 0.1 N H_2SO_4 with 1 ml. caffeine reagent* diluted to 16 ml.	614
5. Same as Solvent 4, but lacking caffeine sodium benzoate component	457
6. Same as Solvent 4, but lacking only caffeine component (*i.e.*, equivalent amount of sodium benzoate added)	471
7. Methyl alcohol	343
8. U. S. P. ethyl alcohol	428
9. Isopropyl alcohol	343
10. *n*-Butyl alcohol	157
11. Isobutyl alcohol	471
12. *n*-Amyl alcohol	257
13. Isoamyl alcohol	400
14. *n*-Hexyl "	200
15. Methylene chloride	28
16. Chloroform	200
17. Carbon tetrachloride	14
18. Ethylene dichloride	243
19. " " 9 volumes + acetic acid, 1 volume	486
20. Propylene dichloride	357
21. " " 9 volumes + acetic acid, 1 volume	257
22. Acetone	114
23. Methyl isobutyl ketone	114
24. " *n*-amyl ketone	128
25. Ethyl acetate	14
26. Amyl "	14
27. *n*-Hexane (skellysolve B)	2
28. Ethyl ether	14
29. Dibutyl ether	14
30. 2,2'-Dichloroethyl ether	583
31. Benzene	2
32. Toluene	14
33. Xylene	43
34. Chlorobenzene	28
35. Trichlorobenzene	43
36. 85% lactic acid	471
37. Tetrachloroethylene	180
38. " 7 volumes + 25% (weight per volume) trichloroacetic acid in absolute ethanol, 1 volume	325

* Described below.

Instrument—Of the three instruments with which the authors have had extensive experience, the Coleman No. 12 photofluorometer has been found to be the most satisfactory. This instrument is subject to the instability inherent in non-balanced circuits, but more than makes up the deficit in superior simplicity and sensitivity. The filter system used by the authors consists of Corning Filter 5113 between the lamp and sample tube, and Corning Filter 3385 between the sample tube and photo element.[1]

Reagents—

$Na_2HPO_4 \cdot 12H_2O$, 5 gm. in 100 ml. of water.

0.1 N (approximate) H_2SO_4.

0.05 N (approximate) NaOH.

10 per cent NaOH.

Ethyl ether.

Solution for intensifying atabrine fluorescence (hereafter termed "caffeine reagent"). This solution is prepared as follows: Dissolve 10 gm. of caffeine sodium benzoate, U. S. P., in 30 ml. of water and 40 ml. of 95 per cent ethanol. Add 20 ml. of diethanolamine, dilute to 100 ml. with water, and mix. Commercial samples of diethanolamine may contain fluorescent impurities. These may be removed by distillation under reduced pressure in an all-glass still. The fluorescent matter remains in the residue.

Standard Atabrine Solution—Dissolve 100 mg. of pure atabrine dihydrochloride dihydrate in water and dilute to exactly 100 ml. Working dilutions should be prepared daily from this stock solution by diluting with 0.2 per cent monosodium phosphate solution so that 1 ml. of the final dilution contains 1 γ of atabrine.

Technique for Blood—Pipette 5 ml. of oxalated whole blood into a 125 ml. Squibb separatory funnel containing 10 ml. of 5 per cent disodium phosphate solution. Add 30 ml. of ether and shake for 3 minutes. Very little emulsion forms. Allow the two layers to separate and tap off the aqueous layer. Wash the ether layer with three portions of about 5 ml. of 0.05 N sodium hydroxide, and once with 5 ml. of distilled water.

To the ether layer add 5 ml. of 0.1 N sulfuric acid and shake for 30 seconds. Collect the acid extract in a 25 ml. glass-stoppered graduated cylinder. Extract the ether with a second 5 ml. portion of acid, adding the second extract to the first. Wash down the stopper with about 2 ml. of water, shake to collect as much as possible of the aqueous layer in the bottom of the funnel, and add this final wash to the collected acid extracts. Dilute to the 15 ml. mark with water. Add 1 ml. of the caffeine reagent, stopper, and mix. The solution is then ready to read in the fluorometer. The standard for comparison consists of a mixture of 1 γ of atabrine (1 ml. of diluted standard) with 10 ml. of 0.1 N H_2SO_4 and 1 ml. of caffeine reagent, diluted to 16 ml. with water. Deduct from all readings the apparent fluorescence of a blank consisting of 10 ml. of 0.1 N H_2SO_4 plus 1 ml. of caffeine reagent diluted to 16 ml. with water.

This method has been applied successfully to human, rat, dog, and rabbit blood. Duck blood, which jellies very badly, must be prepared in a special way. Pipette 5 ml. of oxalated duck blood into a test-tube, add 2 ml. of 10 per cent NaOH, stir with a glass rod, and digest for 30 minutes in a water bath at 80°. Mix the tube contents occasionally with the rod. Cool and transfer the blood digest, with the aid of a minimum volume of water, to a separatory funnel. Continue as already directed.

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[1] Corning Filter 3486 was originally used as the secondary filter, but after a personal communication from Dr. B. B. Brodie, Filter 3385 was adopted.

The alkaline digestion destroys the naturally occurring emulsifying substances so completely that subsequent shakings with immiscible solvents lead to no emulsification whatever. For this reason, many analysts may prefer to assay all blood samples by this alternate method.

The method lends itself to multiple assays. One technician can readily handle twelve funnels set up on one rack.

Technique for Urine—Urine is assayed in the same way as blood, except that it is always necessary to dilute the sample because of the larger quantities of atabrine present. For example, after the oral administration of 100 mg. of atabrine, the average initial 24 hour output of a man is about 0.4 mg. Urine of this concentration should be diluted four or five times before testing. In following the urinary output of persons undergoing chronic dosage, it is often convenient to omit the use of the caffeine reagent, and simply dilute the final sulfuric acid extracts to 25 ml. before comparing with the standard similarly prepared. For this purpose, use 1 ml. of a special standard dilution containing 10 γ per ml.

Technique for Tissues—A weighed aliquot of the tissue is minced in a Waring blendor after being diluted 10 to 40 times with water. 10 ml. of the well mixed suspension are pipetted into a test-tube and 2 ml. of 40 per cent NaOH added. After being mixed with a glass rod the sample is digested for 30 minutes in a water bath at 80°. The contents of the tube are then cooled and transferred with the aid of a little water to a separatory funnel. Ether is added, and the determination completed as already described for blood.

If the preliminary alkaline digestion is omitted, recoveries are 10 to 15 per cent lower than otherwise. This is particularly true of heart and other muscular tissue.

Accuracy of Method—Since these procedures were instituted, late in 1942, periodic rechecks of percentage recoveries from the various tissues show that the loss inherent in the analyses amounts to not more than 2 per cent. This holds true when the atabrine reference standard is subjected to the extraction procedure, together with normal blood, urine, or tissue, as the case may be. However, for the sake of convenience, it is recommended that the reference standard simply be diluted with acid and caffeine reagent as given in the blood technique. The apparent loss then becomes larger (about 4 per cent) but recoveries are still within reasonable limits. A few representative recovery tests are outlined in Table II.

Influence of Temperature on Fluorescence Intensity—It has been pointed out (16) that the temperature of the solvent affects the fluorescence intensity. The quantitative relationship has been investigated and is indicated in Table III.

TABLE II

Recovery Data. Standard Not Subjected to Extraction Procedure

Sample	Atabrine added	Per cent recovered
	γ	
5 ml. whole blood (dog)	0.2	96
5 " " " "	0.6	96
25 " urine (dog)	2.0	97
25 " " "	2.0	97
25 gm. liver "	500.0	96
25 " " "	500.0	96
25 " " "	500.0	97
1 " brain "	10.0	96
1 " heart "	10.0	95
25 " " "	500.0	96
25 " " "	500.0	96
5 ml. whole blood (duck)	0.2	96
5 " " " "	0.6	96
5 " " " (dog)	0.4	96
5 " " " "	0.4	95
5 " " " "	0.4	95

The last five analyses were made after preliminary digestion with sodium hydroxide.

TABLE III

Relative Fluorescence Intensity at Various Temperatures, Expressed As Net Deflection (Galvanometer Divisions), with Instrument at Full Sensitivity

Temperature	0.6 γ in 16 ml. solvent containing caffeine reagent	10 γ in 25 ml water containing 10 ml. 0.1 N H_2SO_4
°C.		
16	52	65
20	49	57
24	48	53
25	45	
26	43	50
28	41	47
32	39	43
36	37	40

These data clearly demonstrate that it is desirable to work at temperatures below 24°. Loss of sensitivity at higher temperatures does not, of course, involve loss of accuracy, so long as both sample and standard are at the same temperature. A question of practical importance is whether the sample heats up to any significant extent during the short time it is held in the fluorometer.

To answer this question, the temperature rise was determined in a sample kept in the instrument for 3 minutes. Readings were taken at the end of every minute. The rise was very regular, amounting to 0.5° per minute. The result was the same, regardless of whether the shutter between the mercury lamp and sample was kept open or shut.

In short, it is unnecessary to attempt to control the temperature of the sample during the fluorometric readings. If the room temperature happens to be above 24°, the sensitivity of the method suffers. The only practical ways to meet this difficulty would be to work in a controlled temperature room, or to employ a controlled temperature bath for the sample tubes. Workers in tropical climates might well give further consideration to this matter.

SUMMARY

A fluorometric assay method for atabrine in body fluids and tissues has been described. The sensitivity is such that a 5 ml. sample of blood containing 0.1 γ of atabrine can be analyzed satisfactorily. The sensitivity is adequate for the analysis of blood levels following normal therapeutic dosage. At the level of 40 γ per liter of whole blood, the recovery loss is about 4 per cent, and can be reduced to about 2 per cent if normal blood is available. The convenience and simplicity of the method should make it especially useful to clinical laboratories. Technicians with no previous specialized training have been able to achieve satisfactory results after only a few days experience.

During the year and a half the method has been used, a large number of determinations have been made on blood, urine, and tissues from various laboratory animals, as well as on human blood and urine. Pharmacological data including these analytical results will be presented elsewhere.

Data are submitted to show the effect of solvent and temperature on the fluorescence intensity of very dilute solutions of atabrine.

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URINARY STEROIDS. USE OF THE PERIODIC ACID REACTION IN THE MEASUREMENT OF NON-KETONIC STEROIDS OBTAINED AFTER VARIOUS TYPES OF HYDROLYSIS*

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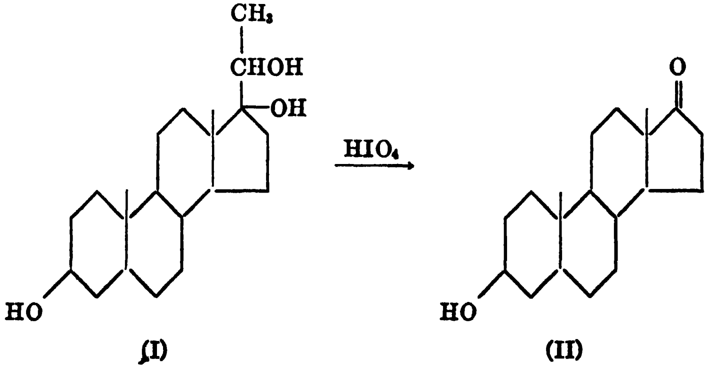
(Received for publication, April 14, 1944)

In a previous paper evidence was presented which showed that, while the conjugated urinary 17-ketosteroid, sodium dehydroisoandrosterone sulfate, was partially destroyed by hydrochloric acid hydrolysis, it could be recovered quantitatively in the free form after barium chloride hydrolysis (1). It was shown further that sodium pregnanediol glucuronidate can be hydrolyzed to free pregnanediol without destruction by means of an enzyme (2). The present paper extends these observations to include measurements of certain free steroids obtained after barium chloride, enzymatic, or hydrochloric acid hydrolysis of extracts of urine from three patients with adrenal cortical virilism.

Convenient methods are available for assaying the 17-ketosteroid content of crude urine extracts. On the other hand, measurement of urinary non-ketonic steroids has been more difficult because, with the possible exception of pregnanediol (3, 4), no sensitive assay procedures for their determination have been available. That urine may contain quantities of interesting non-ketonic alcoholic steroids has been demonstrated by Marrian and Butler who isolated a large amount of 3(α),17,20-pregnanetriol from the urine of a patient with an adrenal cortical tumor (5, 6). Reichstein and others (7) have shown that such steroids with hydroxyl groups at positions 17 and 20 on the steroid molecule (I) may be oxidized readily to



(I) (II)

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* This work was supported by a grant from the Commonwealth Fund of New York.

a 17-ketosteroid (II) by periodic acid. Fieser has recently called attention to the fact that this reaction is relatively specific and reasonably quantitative and that the 17-ketosteroid formed by periodic acid should be determinable by available assay procedures.[1] Accordingly, the present communication reports observations on the application of this reaction to neutral non-ketonic substances obtained after the various forms of hydrolysis mentioned above. The results obtained indicate that the periodic acid reaction should be a valuable adjunct to the measurement of urinary non-ketonic steroids and hence to studies of the excretory transformation products of steroid hormones.

Analytic Procedure

Collection and Extraction of Urine—The procedure employed is similar to that used previously for the recovery of sodium dehydroisoandrosterone sulfate from urine (1). The urine is collected with 200 cc. of *n*-butanol per liter added as a preservative. It is extracted by shaking four times with one-eighth its volume of *n*-butanol in a separatory funnel. The combined butanol extract is washed six times with one-twenty-fifth its volume of N sodium hydroxide solution. The butanol extract is then washed repeatedly with one-twenty-fifth its volume of 0.1 N hydrochloric acid solution until the washings have a pH of between 5 and 7 (red to litmus and to Congo red paper). The extract is finally washed two or three times with one-twenty-fifth its volume of 0.1 N sodium acetate buffer solution (pH 6.0). The pH of these final washings should be between 5.5 and 6.5. The washed butanol extract is then evaporated to dryness in a vacuum distillation apparatus at a temperature not exceeding 25°. The residue is dissolved in a small quantity of methanol (crude unhydrolyzed urine extract). The methanol solution of the extract is stable for several weeks at 0–5°.

Hydrolysis of Crude Unhydrolyzed Urine Extract—Aliquots of the methanol solution of the urine extract may be subjected to barium chloride, enzymatic, or hydrochloric acid hydrolysis as follows:

Barium Chloride Hydrolysis—The procedure employed corresponds closely to that described for sodium dehydroisoandrosterone sulfate (1). An aliquot of the methanol solution of the urine extract is evaporated to dryness at a temperature not exceeding 25°. The residue is dissolved in 100 cc. of 0.1 N sodium acetate buffer solution (pH 6.0) and 15 gm. of C.P. barium chloride are added.[2] The pH of the solution is then adjusted to a value of 5.8 to 6.2 by the addition, as found necessary, of 0.1 N sodium hydroxide solution or of 0.1 N acetic acid solution.[3] This aqueous solution is overlaid with approximately 20 cc. of toluene and is heated on a boiling water bath under a reflux condenser for 4 hours. It is then allowed to cool and is extracted once with the toluene already present and three times with 25 cc. lots of ethyl acetate. The combined toluene-ethyl acetate extract is washed in a separatory funnel three times with 25 cc. portions of N sodium hydroxide solution and 3 times with 25 cc. lots of water.[4] The washed extract is then evaporated to dryness on a steam bath. The residue (barium chloride hydrolysate) is separated into ketonic and non-ketonic fractions with Girard's Reagent T as described below.

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[1] Fieser, L. F., unpublished observations.

[2] The free steroid content of this aqueous solution may be determined by extracting the solution with ether or ethyl acetate prior to heating and hydrolysis. The extract thus obtained is assayed as outlined below.

Enzymatic Hydrolysis—This method of hydrolysis is similar to that devised for the hydrolysis of sodium pregnanediol glucuronidate (2). An aliquot of the methanol solution of the crude urine extract is evaporated to dryness at a temperature not exceeding 25°. Remaining traces of methanol are removed by the addition and subsequent evaporation of small quantities of anhydrous ethyl ether. The dry residue is dissolved in 15 cc. of distilled water. 10 cc. of 0.1 N sodium acetate buffer solution (pH 5) are added and the pH of the resultant solution is determined. If necessary, the pH is adjusted to 5 by the addition of small quantities of 0.1 N sodium hydroxide solution or of 0.1 N acetic acid solution.[3] To the solution at pH 5, 400 mg. of acetone-dried powdered rat liver enzyme are added with brief stirring. After 4 hours incubation at 37°, the mixture is filtered through Whatman No. 2 paper and the insoluble material on the filter is washed three times with 5 cc. portions of water. The original filtrate and the water washings are combined and extracted four times with 10 cc. portions of ethyl acetate.[5] The combined ethyl acetate extract is washed three times with 10 cc. lots of N sodium hydroxide solution and four times with 10 cc. portions of water. In addition, the insoluble material on the filter is extracted on the filter by washing five times with 5 cc. portions of hot ethanol. The ethanol extract thus obtained and the washed ethyl acetate extract mentioned above are combined and evaporated to dryness on a boiling water bath. The residue (enzymatic hydrolysate) is separated into ketonic and non-ketonic fractions, as described below.

Hydrochloric Acid Hydrolysis—An aliquot of the methanol solution of the crude urine extract is evaporated to dryness as described above. The residue is dissolved in 100 cc. of water and 15 cc. of technical hydrochloric acid are added. The mixture is boiled for 10 minutes under a reflux condenser and is then cooled immediately under a stream of cold water. The hydrolyzed mixture is extracted in a separatory funnel four times with 25 cc. portions of ethyl acetate. The combined ethyl acetate extract is washed with N sodium hydroxide solution (25 cc., three times) and with water (25 cc., three times) in a separatory funnel. The washed extract is evaporated to dryness on a boiling water bath. The residue (hydrochloric acid hydrolysate) is separated into ketonic and non-ketonic fractions, as described below.

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[3] Small aliquots were removed for pH determinations. The color chart of indicators of Clark (8) was used.

[4] These washings may be combined and added to the barium chloride hydrolysis solution which, when acidified, may be saved for subsequent hydrochloric acid hydrolysis (see below).

[5] This filtrate plus the water washings may be hydrolyzed subsequently with barium chloride according to the procedure given above after the pH of the solution has been adjusted to 6.

Consecutive Hydrolysis—In experiments designed to obtain maximum yields of hydrolysate, the crude unhydrolyzed urine extract may be subjected to more than one type of hydrolysis as follows: (*a*) enzymatic, followed by barium chloride, followed by hydrochloric acid, or (*b*), enzymatic, followed by hydrochloric acid, or (*c*), barium chloride, followed by hydrochloric acid. Because barium inhibits the enzyme, it is difficult to use enzymatic after barium chloride hydrolysis When the crude urine extract is hydrolyzed consecutively, the hydrolysate formed by each of the procedures is extracted from the aqueous solution as outlined above before another hydrolytic procedure is applied to the partially hydrolyzed aqueous solution.

Separation of Hydrolysate into Ketonic and Non-Ketonic Fractions—The dried residue of the extracts obtained following the various types of hydrolysis is separated into ketonic and non-ketonic fractions with the aid of Girard's Reagent T, according to a procedure described previously (9). The ketonic residue is dissolved in a measured quantity of absolute ethanol preparatory to colorimetric assay. The non-ketonic residue may be weighed and then dissolved in a measured quantity of absolute methanol preparatory to the periodic acid reaction.

Periodic Acid Reaction—This reaction is used to convert certain non-ketonic steroids into 17-ketosteroids. Approximately 5 mg. of non-ketonic material are dissolved in 2.7 cc. of absolute methanol. 0.1 cc. of this solution is removed and saved for colorimetric assay. If after thorough stirring insoluble material is present, it should be removed by filtration. To this solution 0.5 cc. of a freshly prepared 50 per cent aqueous methanol solution containing 70 mg. of periodic acid and then 0.04 cc. of concentrated sulfuric acid are added. After the mixture has stood for 1 hour at room temperature (20–25°), it is transferred to a 250 cc. separatory funnel with 180 cc. of ethyl ether. The resultant ether solution is washed three times with 25 cc. lots of a freshly prepared 10 per cent solution of sodium hydrosulfite in N sodium hydroxide solution. *During each of these washings the separatory funnel should be shaken for about 2 minutes.* The ether solution is now washed once with 25 cc. of N sodium hydroxide solution and four times with 25 cc. portions of water. The washed ether solution is evaporated to dryness. The residue (formed 17-ketosteroids) is dissolved in a measured volume of absolute ethanol preparatory to colorimetric assay.

17-Ketosteroid Assay—The 17-ketosteroid content of each ketonic and non-ketonic fraction is determined colorimetrically by the absolute alcohol-*m*-dinitrobenzene-KOH procedure, as described elsewhere (10). All values are corrected for interfering chromogens by means of a color correction equation (10). 17-Ketosteroids present in fractions which have not been treated with periodic acid are termed *preformed 17-ketosteroids*. The quantity of 17-ketosteroids formed by the action of periodic acid upon non-ketonic material (*formed 17-ketosteroids*) is equal to the 17-ketosteroid content of a given quantity of non-ketonic material after periodic acid treatment minus the 17-ketosteroid content of the same sample before treatment with periodic acid.

Material

A major part of the experimental work reported here has been carried out on extracts of urine obtained from two adult female patients with virilism due to a carcinoma of the adrenal cortex and one 13 year-old girl with virilism due to adrenal cortical hyperplasia. Preliminary measurements have also been made on extracts of urine from normal, young adult men and women. In addition certain studies have been carried out on a sample of Δ^4-pregnene-3-one-17,20,21-triol (m.p. 226–228°) obtained through the courtesy of Dr. L. F. Fieser.

EXPERIMENTAL

Periodic Acid Reaction—In the course of preliminary experimentation it was observed that the efficiency of the periodic acid reaction was influenced by several factors such as the concentration of sulfuric acid used, the duration of the reaction, the concentration of periodic acid used, and the temperature of the reaction. Accordingly, studies designed to define satisfactory conditions for the reaction were carried out.

In these experiments, two types of material were studied. The first was non-ketonic material separated from the barium chloride hydrolysate of urine from a patient with adrenal cortical carcinoma according to the analytic procedure. Colorimetric assay of this material showed that it contained no 17-ketosteroids and that it was essentially free from other chromogens. The second substance used was a sample of the Δ^4-pregnene-3-one-17,20,21-triol. Colorimetric assay of the triol revealed that it had essentially the same chromogenic characteristics as an equimolar quantity of testosterone, which is also a Δ^4-3-ketone. Because of the nature of the color given by the triol in the colorimetric assay reaction before treatment with periodic acid, it was necessary to estimate the quantity of 17-ketone formed by the action of periodic acid on the triol as follows: The extinction coefficients of the colorimetric assay solution at 540 mμ and 420 mμ (E_G, E_B) were determined for a given quantity of the triol before (E'_G, E'_B) and after (E''_G, E''_B) the triol had been treated with periodic acid. The difference in extinction coefficients ($\Delta E = E'' - E'$) was considered to be due to a change in the chromogenic properties of the triol consequent to periodic acid treatment. It was found that the ratio of ΔE_G:ΔE_B was 2.2 to 2.5, values considered to be characteristic of 17-ketosteroids (10). Accordingly, the quantity of 17-ketosteroid formed by the action of periodic acid on the triol was calculated from ΔE_G and ΔE_B in the same manner that the preformed 17-ketosteroid content of urine extracts is determined from E_G and E_B (10). No correction was made for the error of underestimation (approximately 15 per cent) in the colorimetric assay of 17-ketosteroids due to the presence of Δ^4-3-ketosteroid (11).

TABLE I

Effect of Variations in Concentration of Sulfuric Acid Used in Periodic Acid Reaction Mixture on Yield of 17-Ketosteroids from 2 Mg. Samples of Non-Ketonic Material

Except for variations in the sulfuric acid used and except for the fact that the reaction time was 17 hours instead of 1 hour, the reaction was carried out according to the analytic procedure.

Experiment No	H_2SO_4 used	Concentration of H_2SO_4	Yield of 17-ketosteroids
	cc.	*vol. per cent*	*mg.*
1	0.00	0.00	0.02
2	0.01	0.32	0.47
3	0.02	0.65	0.57
4	0.04	1.30	0.68
5	0.08	2.60	0.68

In the following experiments the periodic acid reaction described under the analytic procedure (see above) was varied with relation to, respectively, the concentration of sulfuric acid (Table I; also, reaction time 17 hours instead of 1 hour), the duration of the reaction (Table II), the temperature of the reaction (Table III), and the concentration of periodic acid (Table IV). As shown in the tables, either a 2 mg. sample of non-ketonic material or a 1 mg. sample of the triol was used in each experiment.

It is seen (Table I) that, while periodic acid does form 17-ketosteroids from the non-ketonic material in the absence of sulfuric acid, the yield is greater if the reaction mixture contains from 1.3 to 2.6 volumes per cent of concentrated sulfuric acid. The experiments of Table II show that the

Table II

Effect of Variations in Duration of Periodic Acid Reaction upon Yield of 17-Ketosteroids from Samples of Non-Ketonic Material and of Δ^4-Pregnene-3-one-17,20,21-triol

Except for the duration of the reaction, the reaction was carried out according to the analytic procedure.

17-Ketosteroids from	Experiment No.	Duration of reaction		Yield of 17-ketosteroids
		hrs.	*min.*	*mg.*
2 mg. non-ketonic material	6	0	1	0.00
	7	0	40	0.76
	8	1	20	0.77
	9	2	00	0.85
	10	4	00	0.79
	11	6	00	0.78
	12	17	00	0.71
1 mg. triol	13	0	1	0.29
	14	0	30	0.70
	15	1	00	0.74
	16	2	00	0.70
	17	4	00	0.74
	18	48	00	0.74

Table III

Effect of Variations in Temperature of Periodic Acid Reaction upon Yield of 17-Ketosteroids from 1 Mg. Samples of Δ^4-Pregnene-3-one-17,20,21-triol

Except for variations in temperature, the reaction conditions corresponded to those given under the analytic procedure.

Experiment No.	Reaction temperature	Yield of 17-ketosteroids
	°C.	*mg.*
19	20	0.74
20	30	0.77
21	40	0.70

Table IV

Effect of Variations in Concentration of Periodic Acid Used in Periodic Acid Reaction upon Yield of 17-Ketosteroids from 1 Mg. Samples of Δ^4-Pregnene-3-one-17,20,21-triol

Except for the variations in periodic acid, the reaction conditions corresponded to those of the analytic procedure.

Experiment No	Periodic acid used	Concentration of periodic acid	Yield of 17-ketosteroids
	mg. per sample	*gm. per cent*	*mg.*
22	0	0	0
23	30	0.96	0.65
24	50	1.59	0.76
25	60	1.92	0.74
26	70	2.23	0.85
27	80	2.56	0.86

periodic acid reaction takes place in less than 1 hour and that the 17-ketosteroids formed are stable in the reaction mixture for several hours. The data of Table III indicate that the reaction temperature may be varied at least between 20–30° without influencing the results obtained. Table IV shows that for optimal results the concentration of periodic acid in the reaction mixture should be approximately 2.3 gm. per cent.

The data of Experiments 28 and 29 (Table V) show that measured amounts of the preformed 17-ketosteroids, dehydroisoandrosterone and etiocholanone, were recovered quantitatively after treatment with periodic acid. The value obtained for the triol (Experiment 30) is of interest in that it corresponded reasonably closely to the theoretical. Thus it suggests that the triol was quantitatively converted into a 17-ketosteroid. Experiments 31 and 32 were designed to test the efficiency of the periodic acid oxidation when it was carried out in the presence of urinary non-ketonic material. In these experiments, samples of non-ketonic material were divided into two equal portions To one portion nothing was added; to the other 1 mg. of the triol was added. Both portions were subjected to the reaction and the yield of 17-ketosteroids was determined. It is seen that the yield of 17-ketosteroids from the added triol was essentially equal to that obtained for the triol in pure solution (Experiment 30). Those observations indicate that the non-ketonic materials studied did not contain substances which interfered with the periodic acid reaction.

TABLE V

Recovery of 17-Ketosteroids after Periodic Acid Reaction

Experiment No.	Substance treated*	17-Ketosteroids		
		Determined (*a*)	Theoretical (*b*)	$\frac{(a)}{(b)}$
		mg.	*mg*	
28	2 mg. DHA	2.0	2.0	1.0
29	0.8 mg. ETIO	0.8	0.8	1.0
30	1 mg. triol	0.7	0.8	0.9
31, a	2 " N-K	0.9		
31, b	2 " " + 1 mg triol	1.7	1.7	1.0
32, a	Normal N-K′	0 0		
32, b	" " + 1 mg. triol	0.7	0.8	0.9

* DHA = dehydroisoandrosterone; ETIO = etiocholanone-17; triol = Δ^4-pregnene-3-one-17,20,21-triol; N-K = non-ketonic material used in the experiments of Table II; N-K′ = non-ketonic material derived from the barium chloride hydrolysate of a 24 hour normal urine sample.

Effects of Various Types of Hydrolysis upon Yield of Preformed 17-Ketosteroids and Formed 17-Ketosteroids—Equal aliquots of three crude unhydrolyzed urine extracts from three patients with adrenal cortical virilism were subjected to the various hydrolytic procedures described in the analytic procedure above. Figs. 1 to 3 present the comparative yields obtained.

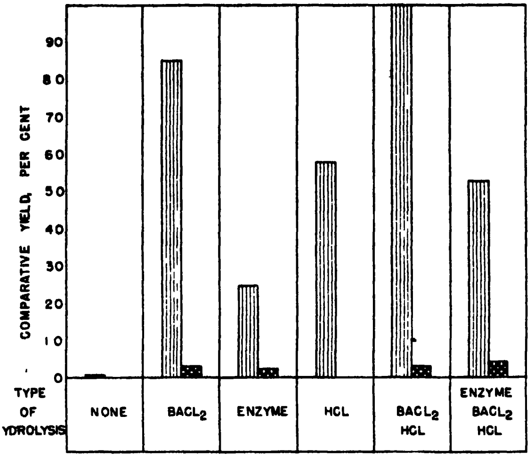

FIG. 1. Effects of various types of hydrolysis upon the yield of preformed 17-ketosteroids and formed 17-ketosteroids. Patient C, virilism due to adrenal cortical carcinoma. The height of the striped columns represents the relative yield of preformed 17-ketosteroids; the height of the adjacent checkered columns represents the relative yield of 17-ketosteroids formed by the action of periodic acid upon nonketonic material. The scale in per cent is given along the left-hand ordinate. The hydrolysis procedure used is noted along the lower margin.

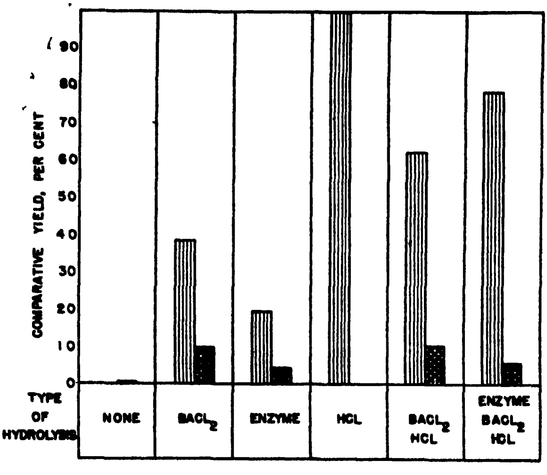

FIG. 2. Effects of various types of hydrolysis on the yield of preformed 17-ketosteroids and formed 17-ketosteroids. Patient S, virilism due to adrenal cortical carcinoma. The arrangement of this figure is similar to that of Fig. 1.

The left-hand columns of Figs. 1 to 3 show that when the crude unhydrolyzed urine extract was not subjected to any hydrolysis the yield of free preformed 17-ketosteroids and of free, formed 17-ketosteroids was poor. This observation suggests that the substances measured were excreted largely if not entirely as water-soluble, conjugated steroids. The columns representing the yields after barium chloride and enzymatic hydrolysis, respectively, show that increased quantities of both preformed and formed 17-ketosteroids were obtained. Hydrochloric acid hydrolysis also resulted in an increase in free preformed 17-ketosteroids, but did not yield any free non-ketonic material from which 17-ketosteroids could be formed by periodic acid. This is of interest because it suggests strongly that the hydrochloric acid procedure not only hydrolyzed, but also altered or destroyed these non-ketonic steroid substances. In two instances (Figs. 1 and 3) the yield of preformed 17-ketosteroids was slightly greater after consecutive barium chloride-hydrochloric acid hydrolysis than after hydrochloric acid hydrolysis alone; in the third instance (Fig. 2), the yield was about one-third smaller. When enzymatic hydrolysis was carried out first, the yield of preformed 17-ketosteroids after further hydrolysis with barium chloride and hydrochloric acid was less than that obtained by hydrochloric acid hydrolysis alone.

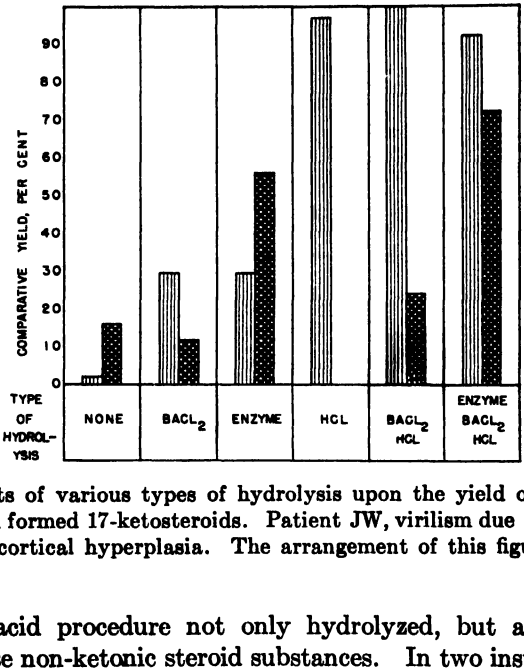

Fig. 3. Effects of various types of hydrolysis upon the yield of preformed 17-ketosteroids and formed 17-ketosteroids. Patient JW, virilism due to congenital bilateral adrenal cortical hyperplasia. The arrangement of this figure is similar to that of Fig. 1.

Daily Excretion of Preformed 17-Ketosteroids and of Formed 17-Ketosteroids—Fig. 4 represents the mg. per 24 hours obtained on the samples of Figs. 1

to 3 after (*a*) barium chloride followed by hydrochloric acid hydrolysis and (*b*) enzymatic, followed by barium chloride, followed by hydrochloric acid hydrolysis. Patient JW had virilism due to adrenal cortical hyperplasia; patients S and C had virilism due to adrenal cortical carcinoma. While the patients with adrenal cortical carcinoma excreted larger quantities of preformed 17-ketosteroids than the patient with adrenal cortical hyperplasia, all the patients had approximately equal values for formed 17-ketosteroids (10 to 16 mg. per day). Preliminary observations by similar methods on normal individuals suggest that they excrete much smaller quantities (approximately 0.4 mg. per day) of the formed 17-ketosteroids. The observations on preformed 17-ketosteroid excretion correspond closely to those obtained by the usual methods of hydrolysis and extraction (12). Further measurements on selected individuals are needed before the exact

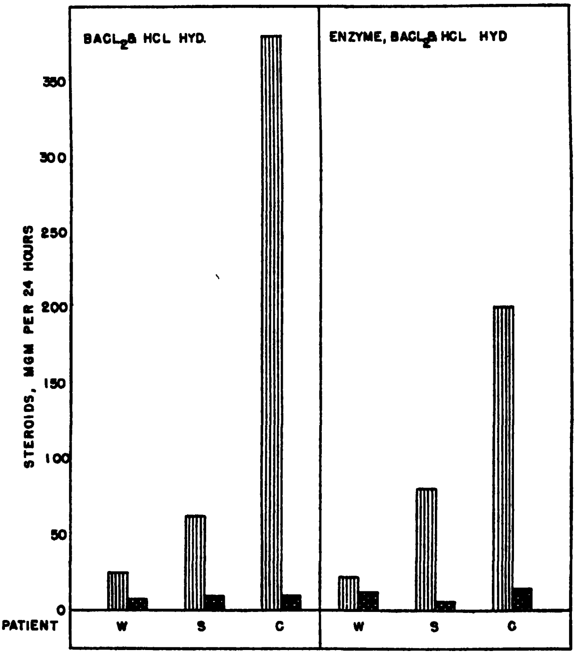

FIG. 4. Daily excretion of preformed 17-ketosteroids and of formed 17-ketosteroids by patients JW (Fig. 3), S (Fig. 2), and C (Fig. 1). The height of striped columns gives the output of preformed 17-ketosteroids, while the height of the checkered columns gives the excretion of formed 17-ketosteroids according to the scale in mg. shown along the ordinate. The results shown in the left-hand portion of the figure were obtained after barium chloride followed by hydrochloric acid hydrolysis, while those of the right-hand portion were obtained after enzymatic followed by barium chloride followed by hydrochloric acid hydrolysis.

clinical and physiologic significance of the formed 17-ketosteroid output can be discussed.

Comments

The periodic acid reaction described above appears to give satisfactory results. In early experiments it was noted that the residue of the ether solution of the periodic acid reaction products tended to char. When this occurred, there was a loss of 17-ketosteroids. Because this charring seemed to be due to residual oxidizing agents, sodium hydrosulfite was added to the sodium hydroxide solution used for washing the ether solution. Although the sodium hydrosulfite introduced no errors, it appears from subsequent experience that this reagent may not be essential, provided the ether solution is shaken very extensively with sodium hydroxide solution.

It is of interest that as much as 0.4 mg. of 17-ketosteroids was obtained from 1 mg. of non-ketonic material after application of the periodic acid reaction (Table II). These formed ketosteroids appeared in the ketonic fraction after treatment with Girard's Reagent T. Furthermore, the colors formed by these ketones in the colorimetric assay reaction corresponded reasonably closely ($E_G:E_B$, 1.6 to 2.2) to those given by pure solutions of 17-ketosteroids (2.2) (10).

The hydrolysis experiments of Figs. 1 to 3 are in keeping with the fact that hydrochloric acid hydrolysis tends to destroy certain conjugated urinary steroids (1, 2). Although the non-ketonic precursor of the 17-ketosteroids formed in the periodic acid reaction has not been identified as yet, it would appear that this substance is quantitatively destroyed by the hydrochloric acid hydrolysis. Presumably the substance in question is characterized by the presence of hydroxyl groups at positions 17 and 20. In this connection it is of interest that the 3(α),17,20-triol of Butler and Marrian was isolated from urine which had not been hydrolyzed with an acid, but which may have been exposed to urinary bacterial enzymes (6).

SUMMARY

A procedure for the quantitative oxidation of 17,20-dihydroxy steroids to 17-ketosteroids has been outlined. Assays of the 17-ketosteroids thus formed and of the usual preformed 17-ketosteroids were carried out on extracts of urine which had been hydrolyzed with the acid of barium chloride, an enzyme preparation, or hydrochloric acid, respectively.

The hydrolysates of the barium chloride and enzymatic procedures contained both preformed 17-ketosteroids and non-ketonic substances which could be assayed colorimetrically after conversion into 17-ketosteroids by periodic acid. On the other hand, while the hydrolysates obtained after hydrochloric acid hydrolysis also contained preformed 17-ketosteroids, they

were devoid of substances from which 17-ketosteroids could be formed with the aid of periodic acid.

It is concluded that human urine contains steroids presumably of adrenal cortical origin which are destroyed by hydrochloric acid hydrolysis, but which may be recovered and measured after the milder barium chloride and enzymatic types of hydrolysis.

We are indebted to L. F. Fieser and S. Lieberman for advice during the course of this work.

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PROTEIN-BOUND IODINE IN ERYTHROCYTES AND PLASMA

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(Received for publication, April 21, 1944)

In 1938 we (1) observed that precipitating blood proteins with cold methyl alcohol and immediately washing the precipitate with cold acetone completely removed added inorganic iodine but retained all of the iodine of added thyroglobulin in the coagulum. We observed that prolonged action of methyl alcohol split off some of the prosthetic group of hemoglobin and tried to avoid this by rapid removal of the methyl alcohol with acetone.

Boyd and Clarke (2) found that a large portion of blood iodine resists extraction with cold alcohol. Perkin and Hurxthal (3) found that when blood proteins are precipitated by cold alcohol and washed with the same, the coagulum contained about 6 to 7 γ of iodine per 100 cc. of normal blood. They found that cold alcohol did not remove iodine from thyroid proteose. It was observed by Cavett, Rice, and McClendon (4) that thyroglobulin repeatedly dissolved in water and precipitated by acetone (and extracted with ether) increased in iodine content from about 0.5 to 0.7 per cent. This may be interpreted as a purification or fractionation of the thyroglobulin, and if cold acetone breaks off iodine from thyroglobulin, one would not expect this result. Davison, Zollinger, and Curtis (5) used acetone to fractionate blood iodine.

In a recent paper Bruger and Member (6) precipitated blood proteins with methyl alcohol and *washed* with acetone and recovered 90 to 93 per cent of the iodine of added thyroglobulin. They precipitated blood proteins with zinc sulfate and sodium hydroxide but *failed to wash* the precipitate and "recovered" 89 to 107 per cent of added "thyroglobulin iodine." In their summary they state that "the recovery of . . . thyroglobulin from the . . . coagulum of whole blood was inadequate when methyl alcohol . . . was used as the protein-precipitating agent." It is not clear to us why they consider 107 per cent better than 93 per cent, both being in error by 7 per cent, and the higher value is to be expected since they failed to wash the precipitate.

It was suggested by Silver (7) that practically no iodine may be demonstrated in erythrocytes, and that plasma or serum should be used for blood iodine determinations. In order to determine the ratio of protein-bound iodine in erythrocytes to that in plasma, oxalated or defibrinated blood was separated in a Swedish (Aktiebolaget) angle centrifuge speeded up by using 135 volts instead of 110 and packed down until refraction planes disappeared.

EXPERIMENTAL

Since the iodine determinations were made by the method of McClendon and Bratton (8), the protein had to be packed into a piece of ⅜ inch Visking sausage casing or Fisher cellophane dialyzing tubing. The methyl alcohol-acetone method has been described previously (1).

The zinc sulfate-sodium hydroxide method was a modification of Somogyi's. 5 cc. of blood (or blood constituent) were mixed with 25 cc. of 2 per cent zinc sulfate and then 5 cc. of 0.5 N sodium hydroxide were added. A 9 inch piece of casing was closed at one end with a filter plug and the other end attached to a slender 100 cc. funnel. The mixture was poured into the funnel and as the filtrate passed through the filter plug, the protein passed into the casing. The protein was washed with 100 cc. of water.

TABLE I

Protein-Bound Iodine (Micrograms per 100 Cc.) in Blood

Blood	Methanol-acetone	Somogyi	$\frac{\text{Methanol}}{\text{Somogyi}}$	Dialysis	$\frac{\text{Methanol}}{\text{Dialysis}}$
Cow	6.4	6.2	1.03	6.2	1.03
Man	6.4			7.0	0.91
"	7.4			7.4	1.00
"	6.0			5.6	1.07
"	5.8			7.4	0.78
"	9.4			8.0	1.17
"	7.2			7.0	1.03
"	7.6			7.8	0.97

For dialysis, 5 cc. of blood were introduced into 5 inches of casing which was tied at both ends and immersed in hot water in a 1 liter beaker, where it remained 12 hours. A smaller number of failures by leakage occurred with hot water than with cold; otherwise the results were the same, and the fractionation is by dialysis and not by heat. In our early experiments dialysis for 3 days was employed, but this is not practical in routine experiments and we are interested in testing such methods even though a slight error may be involved.

It may be seen from Table I and from the blood plasma and erythrocytes of Dog A in Table II that the methyl alcohol method of precipitating blood proteins followed by acetone washing gives similar results to the dialysis and Somogyi methods.

It may be seen from Table II that the concentration of protein-bound iodine in the erythrocytes is almost as high as in the plasma.

It seems evident that if one analyzes only plasma or serum he loses nearly half his material, with no advantage if the iodine is to be analyzed by a closed method. The ash of erythrocytes contains ferric iron and, if it is acidified, iodine escapes as elemental iodine. Therefore in an open method of analysis it may be of advantage to use plasma.

TABLE II

Protein-Bound Iodine (Micrograms per 100 Cc.) in Plasma and Erythrocytes

	Plasma	Erythrocytes
Dog A, dialysis.	5.4	4.5
" " Somogyi.	5.4	5.6
" " methanol-acetone	4.2	5.0
" B, "	4.5	5.5
Man A, "	4.2	5.2
" B, "	5.0	4.9
Cow, "	6.8	6.4
Horse, "	9.2	7.4
Rabbit A, "	11.1	11.2
" B, "	6.6	5.9
Cat serum, "	7.6	7.2
Average	6.36	6.26

DISCUSSION

Iodine analysis of blood or other tissue is still a difficult problem and therefore we made all our determinations in quadruplicate. When a satisfactory technique is mastered, the reduction of the iodine intake to a minute quantity is necessary in order to show any relation between total blood iodine and thyroid function. The analyst is not always able to determine whether the patient has eaten an oyster, for instance. Therefore fractionation is desirable.

The way the blood iodine is fractionated affects the ease of subsequent analysis. If a wet ashing method is used, a high water content of the protein fraction may merely add to the difficulty of washing, but if a dry ashing method is used, this water must be evaporated before ashing. This last process may be avoided by precipitating the proteins in a more volatile medium.

SUMMARY

1. With the methyl alcohol method of precipitating blood proteins practically the same amount of iodine is retained as in the Somogyi method,

provided adequate washing of the precipitate is performed, with acetone in the first instance and water in the second.

2. There is no significant difference between the results with the methanol-acetone method and 12 hours dialysis.

3. The protein-bound iodine in the erythrocytes and plasma is of the same order of magnitude.

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MANOMETRIC, TITRIMETRIC, AND COLORIMETRIC METHODS FOR MEASUREMENT OF UREASE ACTIVITY

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(Received for publication, May 16, 1944)

Van Slyke and Cullen (1, 2) developed a titrimetric method for measuring urease activity in which the enzyme acted under such conditions (pH 6.6 to 7.4, 0.5 M phosphate, urea concentration 5 per cent) that the amount of ammonium carbonate formed in 15 minutes was directly proportional to the amount of urease.

The urease preparations now used are several times more active than those of Van Slyke and Cullen (1). Although prepared by Van Slyke and Cullen's acetone precipitation method (1), that procedure is now applied to a water extract of jack beans, instead of the soy bean extract used by Van Slyke and Cullen. Jack beans, as shown by Mateer and Marshall (3), are much richer in urease.

The activity measurement requires modification for these more active preparations of urease. The enzyme concentration must be cut down, or so much urea will be hydrolyzed that the pH will rise above 7.4, and diminish the activity of the enzyme. However, if this effect is prevented by using lower concentrations of the urease, not enough jack bean protein is present to stabilize the enzyme. It appears that a certain concentration of protein is necessary to protect the enzyme from partial inactivation in the presence of the high concentration of urea used in the standardization. Also, when the standardization is carried out by the gasometric method in the Van Slyke-Neill chamber (4–6), inactivation in protein-poor solutions is accelerated by the mercury which is present.

These difficulties have been overcome by dissolving the urease in a 5 per cent solution of egg albumin, by rinsing the Van Slyke-Neill chamber with albumin solution before each analysis, and by maintaining a concentration of 1 per cent albumin in the reacting urea-urease mixture.

Three procedures will be described, gasometric, titrimetric, and colorimetric, respectively. In the first the enzyme activity is measured by the rate of CO_2 formation. In the second the Van Slyke-Cullen method for measuring urease activity by the rate of NH_3 formation is modified to meet present conditions. In the third procedure the time is measured which is required for enough ammonium carbonate to form to raise the pH of a phosphate buffer from 6.8 to 7.7, the end-point being determined by the color of phenol red indicator.

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THEORETICAL

Van Slyke and Cullen (7) showed that at constant pH the urease in its kinetics acts in accord with the conception that decomposition of substrate occurs in two successive stages, (1) combination of substrate and enzyme and (2) decomposition of the combined substrate. The combination stage was shown to follow the mass action equation, and to require a time interval that is shorter as the urea concentration is greater. When the urea concentration is great enough, the time interval required for combination becomes insignificant compared with the time required for decomposition. With such urea concentration, the enzyme acts at maximum velocity because approximately 100 per cent of it is continually combined with substrate and therefore actively decomposing the latter.

The kinetics of the reaction were expressed by the equation

$$E = \frac{1}{t}\left(\frac{1}{K_1}\log\frac{a}{a-x} + \frac{x}{K_2}\right) \tag{1}$$

E represents enzyme concentration, t the time of reaction, a the initial substrate concentration, and x the decrease in substrate concentration at time t. (In the case of urea, the concentration of either the CO_2 or NH_3 of the $(NH_4)_2CO_3$ formed is a measure of x.) The constant K_1 is either the velocity constant of the reaction by which enzyme and substrate combine (7, 8), or the equilibrium constant of the reversible reaction, enzyme + urea $\leftrightarrows$ enzyme-urea combination (8), whereas K_2 is the velocity with which urea in the urea-enzyme combination is hydrolyzed to NH_3 and CO_2.

Equation 1 was developed in nearly the same form by Michaelis and Menten (9) from a study of invertase. It has been found to be general for hydrolytic enzymes and for some enzymes of other types (8).

When a urea concentration, a, is used which is sufficiently great, and a reaction interval short enough to make x sufficiently small, the fraction $a/(a-x)$ approaches unity, log $a/(a-x)$ approaches zero, $1/K_1$ log $a/(a-x)$ becomes insignificant in comparison with x/K_2, and the equation simplifies to

$$E = \frac{x}{K_2 t} \tag{2}$$

Under these conditions, with constant time t, the amount of CO_2 or NH_3 formed, x, is directly proportional to the urease concentration, E. Van Slyke and Cullen (1) found that these conditions were approximated when the urea concentration was 5 per cent, 0.5 M phosphate of pH 6.6 was used to keep the pH at 6.6 to 7.4, and sufficient soy bean urease was used to decompose not over 0.1 of the urea ($x = 0.1a$) in the 15 minute interval of the reaction.

In the present manometric and titrimetric procedures advantage is also taken of conditions which validate Equation 2; a constant t interval is used, and E is directly proportional to x, the $(NH_4)_2CO_3$ formed.

In the colorimetric procedure advantage is taken of the fact, demonstrated by Van Slyke and Cullen (7), that the *time* required to produce a given amount of ammonium carbonate varies *inversely as the urease* concentration. This inverse proportionality is a general law of enzyme action, which appears to hold when an enzyme is stable for the observation period used (8). The effects of products on the reaction velocity do not alter the accuracy of the inverse relation, because for decomposition of a given amount of substrate the same amounts of products are formed, with the same effects on the velocity. Hence when this timing procedure is applied to urease, the necessity disappears for restraining the pH shift caused by ammonium carbonate to a short range.

I. MANOMETRIC PROCEDURES

Two manometric procedures, A and B, will be described.

In Procedure A the urease action and CO_2 determination both occur in the Van Slyke-Neill chamber.

In Procedure B the urease action occurs, not in the gas chamber, but in a separate flask, from which an aliquot of the solution is pipetted into the Van Slyke-Neill chamber to determine the CO_2 formed.

Procedure B can be used when the mercury in the Van Slyke-Neill chamber is so contaminated with traces of other metals that formation of heavy metal ions would be rapid enough to cause appreciable inactivation of urease if the digestion were carried out in the chamber, even in the presence of 5 per cent albumin. Procedure B is a little less convenient than Procedure A, but equally accurate.

Apparatus

The only special apparatus required is the Van Slyke-Neill (5, 6) manometric apparatus.

All glass apparatus that comes in contact with the reagents is cleaned with nitric acid in order to make certain that no traces are present of salts of heavy metals. These, particularly Hg^{++} even in minimal amounts, may inactivate the urease.

Reagents—

0.65 M phosphate buffer. 5.66 gm. of K_2HPO_4 (0.0325 mole) and 4.49 gm. of $NaH_2PO_4 \cdot H_2O$ (0.0325 mole) are made up to 100 cc. The solution has a pH of 6.4. 0.5 cc. of this diluted to 6.5 cc. in the standardization mixture gives 0.05 M phosphate and pH 6.8 (glass electrode).

Urea solution. 3 gm. per 100 cc. (0.5 M).

2 N lactic acid.

Caprylic alcohol.

Brom-thymol blue, 0.1 per cent.

10 per cent egg albumin in water.

Urease solution containing 50 mg. of egg albumin per cc. If the urease to be tested has activity of the order found in the dry preparation prepared from jack beans by the acetone method of Van Slyke and Cullen (1), a 0.1 per cent solution is prepared. 0.500 gm. of urease is mixed with 3 cc. of water until suspension is uniform; the mixture is then diluted with water to 50 cc. 5 cc. of this 1 per cent urease solution and 25 cc. of the 10 per cent egg albumin are mixed and diluted to 50 cc., giving 0.1 per cent urease in 5 per cent albumin.[1]

1 cc. of this 0.1 per cent solution will hydrolyze enough urea to give in 5 minutes 100 to 500 mm. of CO_2 pressure at 2 cc. volume, if the urease is a preparation of good activity prepared by the acetone method from jack beans. If the urease is of such high or low activity that the CO_2 formed gives a pressure outside the range 100 to 500 mm., it is advisable to use a urease solution of concentration lower or higher than 0.1 per cent.

Before an activity determination is started all solutions should be at the same temperature as the water jacket of the manometric chamber.

MANOMETRIC PROCEDURE A

Preparation of Gas Chamber—The chamber of the apparatus is cleaned and is rinsed once with 1 cc. of 10 per cent egg albumin to remove any traces of mercury ions that may be present.

Addition of Reagents—0.5 cc. of the 0.65 M sodium phosphate buffer is placed in the cup of the Van Slyke-Neill chamber, and is run into the chamber until the cup is emptied, but the capillary below it is filled with solution. Then 1 drop of 0.1 per cent brom-thymol blue, followed by a drop of caprylic alcohol, is run into the capillary. The inflow of the reagents is regulated by the cock which connects the chamber with the leveling bulb.

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[1] Occasional preparations of urease when diluted to 0.1 per cent lose activity fairly rapidly even in the presence of 5 per cent albumin. In one instance the activity of a 0.1 per cent solution of Squibb's "double strength" urease dropped 31 per cent in 4 hours. This loss was prevented completely by adding, to the 0.1 per cent urease-5 per cent albumin solution, glutathione or sodium thioglycolate to a concentration of 0.05 per cent. Protection by KCN, cysteine, glycine, or gum arabic was incomplete (10–13). Therefore the addition of glutathione or thioglycolate to the albumin is advisable when a preparation which is not adequately stabilized by albumin alone is being standardized, or when the stability of the preparation is unknown. Most preparations are stable in albumin without glutathione, and all the data reported in this paper were obtained without the addition of glutathione.

About 0.5 cc. of mercury is now placed in the cup and 5.0 cc. of 3 per cent urea solution are run into the chamber from a stop-cock pipette with rubber-ringed tip ((6) p. 125, Fig. 3). The capillary is sealed by filling it with part of the mercury in the cup, the cock is closed, and the mercury in the chamber is lowered and raised three times to mix the solution.

In the same way 1 cc. of 0.1 per cent urease solution in 5 per cent egg albumin is measured into the chamber, the inflow being controlled by the stop-cock leading to the leveling bulb, so that the urease is layered above the denser substrate solution.

Digestion—As soon as the urease solution is in the chamber, the capillary of the upper cock is sealed with mercury and closed, a stop-watch is started, and the mercury in the chamber is lowered four times to mix thoroughly the contents of the chamber.

1 cc. of 2 N lactic acid is placed in the cup. The temperature in the water jacket of the manometric chamber is read at the mid-point of the 5 minute incubation period. When the stop-watch indicates exactly 5 minutes of urease action, 0.5 cc. of the 2 N lactic acid is run into the chamber and mixed with the solution there, stopping the action of the urease.

Determination of CO_2—The capillary of the cock is sealed with mercury, and the CO_2 is extracted from the acidified solution by evacuating and shaking as in blood CO_2 determinations. After 2 minutes extraction the gas volume is brought to 2 cc. by the technique described by Van Slyke and Neill ((14) p. 277–278), and manometer reading p_1 is taken. Then the cock leading to the leveling bulb is opened and 0.5 cc. of 5 N NaOH is added under slight negative pressure, to absorb the CO_2. The aqueous meniscus is returned to the 2 cc. mark, and manometer reading p_2 is taken (see (14) p. 284–285).

Blanks—Duplicate blank determinations are made in which the urease solution is replaced by 5 per cent egg albumin solution without urease. The $p_1 - p_2$ reading of the blank is the c correction. It is well to run the blank immediately before the determination with urease in order to add to the certainty that Hg ion is removed from the apparatus before the activity determination itself is started.

Calculation of Sumner Urease Units

Sumner and Graham (15) proposed, as a unit of urease activity, the amount capable of producing 1 mg. of ammonia nitrogen in 5 minutes at 20° in a urea solution buffered with phosphate. The definition implies action of urease under specific conditions which (*a*) maintain the pH and substrate concentration within the limits compatible with approximately maximum enzyme activity, and (*b*) protect the enzyme from inactivation. The unit is convenient, for the reason that Sumner found the number of units per mg. of preparation to equal approximately the percentage of crystalline urease present. It appears also proper to retain in the nomenclature the name of Sumner, whose urease was the first of the crystallized enzymes.

In the calculation the CO_2 pressure, P_{CO_2}, which the CO_2 from the generated ammonium carbonate exerts at 2 cc. volume, is calculated as

$$P_{CO_2} = p_1 - p_2 - c \tag{3}$$

where p_1 and p_2 are the manometer readings obtained in the analysis, and c is the value of $p_1 - p_2$ obtained in the blank analysis.

The number of Sumner units of urease present *per mg. of the urease powder* tested is calculated as

$$\textit{Sumner units per mg.} = \frac{5 \times P_{CO_2} \times A}{mg. \times min.} \tag{4}$$

A is the factor in the "A" column of Table I, *mg.* represents the number of mg. of urease preparation present in the digest, and *min.* represents the number of minutes the urease acts before the action is stopped by addition of the lactic acid.

When, as in the routine procedure outlined, *mg.* = 1 (1 cc. of 0.1 per cent urease), and *min.* = 5, the calculation simplifies to

$$\textit{Sumner units per mg.} = P_{CO_2} \times A \tag{5}$$

The *concentration* of urease in Sumner units per cc. of the urease solution added (*not* per cc. of digest) can be calculated by replacing *mg.* by *cc.* in Equations 4 and 5, *cc.* in this case indicating cc. of urease solution added (*cc.* = 1 in the procedure outlined above).

When many urease activities are to be determined, it is convenient to plot the factors of Table I on a graph of semilogarithmic paper, with factors plotted on the logarithmic ordinate; the resulting curve is nearly linear.

Derivation of Calculation Formulae—The general equation for calculating the number of Sumner units present in a digest is

$$\textit{Sumner units} = \textit{mg. ammonia N formed} \times \frac{5}{min.} \times \frac{\textit{activity at } 20°}{\textit{activity at } t°} \tag{6}$$

The ratio, *activity at 20°*: *activity at t°*, refers to the relative rates (under the conditions specified above) at which urease acts at 20° and at t°, the temperature at which the test is carried out.

The ammonia nitrogen formed is calculated by multiplying P_{CO_2} by $0.02802F$, where F is the factor used to calculate mM of CO_2 per liter ((16) p. 142) from P_{CO_2} when the sample is 1 cc., the volume of solution, S, in the chamber is 7 cc., and the

CO_2 pressure is measured with the gas volume, a, at 2 cc. The factor thus obtained is multiplied by the temperature correction for activity in column C, to obtain the factor in column A of Table I. $P_{CO_2} \times A$ therefore represents

$$mg.\ ammonia\ N\ formed \times \frac{activity\ at\ 20^\circ}{activity\ at\ t^\circ}$$

Substituting $P_{CO_2} \times A$ for this product in Equation 6 and dividing by the mg. of urease give Equation 4.

MANOMETRIC PROCEDURE B

Digestion—A 25 cc. Erlenmeyer flask is provided with a 1-hole rubber stopper, the hole of which is closed by a glycerol-lubricated glass plug. All reagents are brought to the same temperature, t_1, approximately the temperature of the room. Into the flask are measured and mixed 10 cc. of 3 per cent urea, 1 cc. of 0.65 M phosphate buffer, and 1 drop of brom-thymol blue solution. Then 2 cc. of the urease solution are added, the flask is stoppered, and the reagents are mixed, while a stop-watch is started.

After sufficient ammonium carbonate has been formed (when the indicator turns bluish green, usually 4 to 8 minutes), the glass plug is withdrawn from the stopper, 2 drops of concentrated NaOH (18 or 19 N) are added from a glass capillary inserted through the hole, and the plug is at once replaced. The alkali can thus be added without escape of any CO_2. (The technique has been detailed for use in determination of urea in urine ((4), (14) p. 366).) The alkali serves two purposes: it stops the action of the urease, and it makes the solution reabsorb the small amount of CO_2 that escapes into the gas space of the flask during the digestion.

The alkali is at once mixed with the solution, and at the same moment the time is recorded as the end of the period of the enzyme's action. Except during removal of samples for analysis, the flask is thereafter kept stoppered to prevent absorption of atmospheric CO_2 by the alkalinized solution.

Determination of CO_2—A 5 cc. portion of the 13 cc. of solution is transferred to the manometric chamber, by a pipette with a rubber-ringed tip, and 0.7 cc. of 2 N lactic acid, followed by a drop of caprylic alcohol, is added. The CO_2 is extracted and p_1 and p_2 are read as in Procedure A. The temperature of the water jacket of the Van Slyke-Neill chamber is recorded as t_2.

Blank—The value of c is the value of $p_1 - p_2$ obtained in a blank analysis done in the same manner as the determination, except that 2 cc. of 5 per cent albumin solution without urease replace the 2 cc. of urease-albumin solution.

Calculation—P_{CO_2} is calculated as in Equation 3, Procedure A. Thence

$$(7) \qquad Sumner\ units\ per\ mg. = \frac{10 \times P_{CO_2} \times B \times C}{mg. \times min.}$$

B and *C* are the factors under columns *B* and *C* in Table I. Their product in column *D* can be used when the digestion and analysis are performed at

TABLE I

Factors for Calculation of Results by Manometric Procedures A and B

$a = 2, S = 7, i = 1.017$

Temperature	Factors for Procedure A *A*	Factors for Procedure B		
		B for t_2 Factors for calculating NH$_3$-N from P_{CO_2}	*C* for t_1 Ratio $\frac{\text{activity at } 20^\circ}{\text{activity at } t^\circ}$	$D = B \times C$ can be used when t_1 and t_2 are equal
°*C.*				
5	0.00921	0.00499	2.31	0.01153
10	0.00680	82	1.76	0.00850
15	0.00486	68	1.30	0.00608
16	57	65	1.23	0.00572
17	31	61	1.17	39
18	06	58	1.11	08
19	0.00382	55	1.05	0.00479
20	61	53	1.00	53
21	40	50	0.950	27
22	21	47	0.900	02
23	04	45	0.860	0.00381
24	0.00287	42	0.818	61
25	72	39	0.779	42
26	58	37	0.743	25
27	44	34	0.709	08
28	31	32	0.676	0.00292
29	20	30	0.645	77
30	09	28	0.617	64
31	0.00199	25	0.592	52
32	90	23	0.568	40
33	81	21	0.545	29
34	73	19	0.525	20
35	66	17	0.511	13
40	34	05	0.417	0.00169

the same temperature, so that $t_1 = t_2$. The "*mg.*" in Equation 7 is the mg. of urease preparation added to the digestion flask; not as in Equation 4, the mg. in the manometric chamber.

When, as in the usual analysis outlined above, "*mg.*" = 2, and *min.* = 5, the calculation simplifies to

$$\text{(8)} \qquad \textit{Sumner units per mg.} = P_{CO_2} \times B \times C$$

If digestion and manometric analysis are performed at the same temperature, so that $t_1 = t_2$, the calculation further simplifies to

$$\text{(9)} \qquad \textit{Sumner units per mg.} = P_{CO_2} \times D$$

To calculate activity *concentrations* in terms of Sumner units *per cc. of the added urease solution*, substitute 2 for "*mg.*" in Equation 7, or, when the time is 5 minutes, use Equation 8 or 9 unchanged.

To obtain the factors in column B, Table I, the factors for calculating mg. of urea nitrogen per liter of 1:10 blood filtrate from analysis of 5 cc. samples, with $S = 5.7$ cc. and $a = 2$ cc. ((4) p. 712; or (14) p. 369) are multiplied by 0.0065 to give the mg. of ammonia nitrogen in half of the 13 cc. of digest, and thereby to permit the use of the convenient constant, 10, in Equation 7.

SPECIAL POINTS IN MANOMETRIC PROCEDURES A AND B

Modifications of Procedure to Obtain CO_2 Pressures within the Desirable Range—The desirable range of P_{CO_2} is from 200 to 500 mm. If the CO_2 pressure is much less than 200 mm., the measurements are less accurate. If such a low pressure is obtained by the routine procedure, more accurate results will be obtained by repeating the determination with a urease solution containing 2 mg. or more of the urease preparation per cc., instead of the routine 1 mg. If Procedure B is used, in which the digestion is carried out in the absence of mercury, the desired increase in CO_2 yield can be obtained, without use of a more concentrated urease, by lengthening the duration of the digestion period to 10 or 15 minutes.

If the CO_2 pressure is over 500 mm., so much ammonium carbonate will have been formed that the pH has been raised above 7.3. As shown by Fig. 1, when the pH exceeds 7.3 the velocity of the urea hydrolysis begins to decrease, and the rate of CO_2 formation ceases to be accurately proportional to the urease activity. In such a case the urease solution is diluted with 5 per cent albumin so that the concentration of urease preparation is 0.5 mg. or less per cc., and the activity is redetermined with the diluted solution.

When any of these changes are made from the routine 5 minute digestion or 0.1 per cent urease solution, the calculations must be made by Equation 4 (Procedure A) or by Equation 7 (Procedure B), and not by the simplified Equations 5, 8, or 9, that can be employed for the routine conditions.

Effects of Mercury in Procedure A—The longer the apparatus stands between duplicate determinations the greater is the amount of mercury ion formed in the chamber, and consequently the lower will be the apparent activity of the enzyme measured by Procedure A, unless the apparatus is thoroughly shaken with egg albumin solution before the determination. The prescribed single rinsing with the egg albumin solution is adequate to reduce the retarding ions to an insignificant concentration only when the chamber is washed with the albumin immediately before the first determination of a series, and when the subsequent determinations follow one another in close succession. Should the work be interrupted for more than 10 minutes, it is advisable to shake up the mercury in the chamber for 2 minutes with a few cc. of albumin solution before proceeding with analyses.

As shown in Fig. 3, the amount of urea split is almost proportional to the time of action of the enzyme. The very slight decrease in activity with time indicated in Fig. 3 is due largely to inactivation of the enzyme by mercury ion forming in the chamber after the start of the incubation. The retardation by this factor is so small that it can be neglected for most purposes, provided the concentration of enzyme used is not so low as to necessitate prolongation of the incubation period to more than 10 minutes. The purer the mercury in the apparatus the slower is the formation of inactivating ion. In general it is preferable to keep the digestion period of Procedure A within 5 minutes and use increased amounts of urease if the activity is too low to give 200 mm. of P_{CO_2} with 0.1 per cent urease solution.

Uniformity of Urease Solution or Suspension—When jack bean urease prepared by the acetone precipitation method is mixed with water, part of the material dissolves and part forms a milky colloidal suspension. For accurate activity determinations it is necessary to make the suspension uniform, as the particles appear to be active in the digestion mixture. In 50 per cent glycerol the material forms a translucent, more uniform solution, but duplicate activity measurements on the glycerol solution agreed less closely than those on water suspensions; presumably the viscosity of the glycerol solution made accurate pipetting difficult. To obtain a uniform mixture of the enzyme with water, the water is added in small portions, the first being just enough to wet the powder. Each portion of water is thoroughly stirred into the mixture before another portion is added, until most of the water required to make a 10 per cent mixture has been added. The mixture is then made up to volume, and more dilute solutions are prepared from it. Before a portion is withdrawn, of either the 10 per cent or the more dilute solutions, the solution is stirred with a footed rod.

Effect of Temperature—An error of 1° in recording the temperature at which the digestion occurs will cause an error of approximately 7 per cent in the estimated activity.

II. ACTIVITY MEASUREMENT BY THE AERATION-TITRATION METHOD

Apparatus

The only special apparatus required is the series of aeration tubes described by Van Slyke and Cullen ((1), (14) p. 548). It is important that the test-tubes in which the urease acts, and the fittings for these tubes, shall not be allowed to touch Nessler's reagent or other mercury solution. If such contact occurs, the tubes and fittings must be cleaned thoroughly with nitric acid.

Reagents—

Urea-buffer mixture of pH 6.8. 3 gm. of urea, 1.10 gm. of K_2HPO_4, 0.85 gm. of $NaH_2PO_4 \cdot H_2O$, diluted to 100 cc. with water.

Caprylic alcohol.

Brom-cresol green, 0.1 per cent.

10 per cent egg albumin.

Boric acid, 4 per cent (approximately).

0.01 N sulfuric acid.

Saturated potassium carbonate, 90 gm. per 100 cc.

Urease solution, 0.2 per cent in 5 per cent egg albumin, is prepared by diluting 1 cc. of 5 per cent urease and 12.5 cc. of 10 per cent albumin[1] to 25 cc. with water.

Procedure—The urea-buffer solution is brought to room temperature, the temperature of the solution is recorded, and 5 cc. portions are measured into each of two aeration tubes (Tube A of Van Slyke and Cullen (1), Fig. 2; (14) Fig. 74, p. 548). One tube serves as the digestion tube for the action of the urease; the other tube serves for the blank, to determine the ammonia present in the reagents.

Two receiving tubes (Tube B of Van Slyke and Cullen (1, 14)) are charged each with 25 cc. of 4 per cent boric acid solution and 2 drops of brom-cresol green solution.

Into each of the two tubes with urea-buffer solution are dropped 2 drops of caprylic alcohol, and into each boric acid tube 1 drop.

To the *digestion tube* 1 cc. of the 0.2 per cent urease solution is added, the solutions are mixed with the inlet aeration tube, and the exact time of mixing is recorded. The stopper is set in place and digestion is allowed to proceed exactly 15 minutes. If the room temperature is variable, the digestion tube is stood, before and during digestion, in a large beaker with water at recorded temperature. At the end of the 15 minute period the stopper is lifted momentarily and 10 cc. of saturated K_2CO_3 solution are quickly added and mixed with the solution. The stopper is quickly set in place to prevent escape of ammonia, and the tube is connected with its boric acid receiving tube.

During the 15 minute period of enzyme action 10 cc. of saturated K_2CO_3 solution are added to the *blank* and mixed with the urea-buffer solution. *After* the carbonate is mixed with the urea, 1 cc. of 0.2 per cent urease solution is added, dropwise with constant stirring. The alkaline carbonate prevents any action by the enzyme in the blank tube.

After the digestion has been ended both the digestion tube and the blank are connected with their boric acid receiving tubes, and the ammonia formed is aerated into the acid as directed by Van Slyke and Cullen ((2), (14) p. 550), by passage of about 75 liters of air through the train. Room air is freed from traces of ammonia by passage through 5 per cent sulfuric acid placed in an extra tube first in the series.

To assist in ascertaining the end-point of the titration a control solution is prepared by measuring 25 cc. of the 4 per cent boric acid and 25 cc. of water into an aeration tube of the same dimensions as the receiving tubes, and adding 2 drops of the brom-cresol green solution. The solution in each receiving tube is titrated with 0.01 N H_2SO_4 or HCl until the color approaches that of the control. Then enough water is added to bring the volume to approximately 50 cc. and the 0.01 N acid is added until the titrated solution matches the control.

If the titration requires less than 10 or more than 25 cc. of 0.01 N acid, the determination is repeated with a different concentration of urease.

Calculation—The cc. of 0.01 N acid used to titrate the ammonia from the digestion tube is indicated as *acid*, and the 0.01 N acid required to titrate the blank as *blank*. The mg. of urease in the digestion tube are indicated as *mg.* *C* is the factor (Table I, column *C*) which corrects for the difference in enzyme activity at 20° and the activity at room temperature. Then

$$\textit{Sumner units per mg.} = \frac{0.0467 \times C\ (\textit{acid minus blank})}{\textit{mg.}}$$

When 2 mg. of urease are used (1 cc. of 0.2 per cent) the calculation is

$$\textit{Sumner units per mg.} = 0.02335 \times C\ (\textit{acid minus blank})$$

The procedure of absorbing the ammonia in 4 per cent boric acid solution and titrating back with standard sulfuric acid to the initial pH of the boric acid, introduced for Kjeldahl analyses by Meeker and Wagner (17) is used above in place of receiving the ammonia in 0.01 or 0.02 N HCl solution and titrating the excess with alkali, as done by Van Slyke and Cullen (7, 8). This substitution in the Van Slyke-Cullen aeration method has previously been used by Sobel *et al.* (18), with 2 per cent boric acid as the receiving solution. Van Slyke and Hiller[2] have found, however, that the passage of 75 liters of air through a 2 per cent boric acid solution, containing 1 or 2 mg. of ammonia, drives off about 1 per cent of the ammonia. If 4 per cent boric acid is used, instead of 2 per cent, the loss is diminished to about 0.3 per cent. Unless precision greater than 1/300 is needed, the convenience of the boric acid method makes its substitution worth while. When the end-point is reached in the boric acid titration, it is essential that the titrated solution and the control used for comparison be within about 2 cc. of the same volume; a difference of 2 cc. in volume makes a change of about 0.03 in the pH of the end-point. With moderate care the titration error due to the end-point can be kept within 0.02 cc. of 0.01 N sulfuric acid.

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[2] Van Slyke, D. D., and Hiller, A., unpublished data.

III. ACTIVITY MEASUREMENT BY THE COLORIMETRIC TIMING METHOD

This procedure is based on the general law of inverse proportionality between enzyme concentration and the time required for a given amount of product to form, which has been discussed in the theoretical part of the introduction. Van Slyke and Cullen (7) showed that this law holds for urease. In the present timing method the urease acts on urea in a phosphate buffer solution. The ammonium carbonate formed reacts with the acid phosphate in the buffer.

$$CO_3^{=} + H_2PO_4^{-} \leftrightarrows HCO_3^{-} + HPO_4^{=}$$

The result is an increase in the ratio, $HPO_4^{=}:H_2PO_4^{-}$, and in pH, which depends on the amount of ammonium carbonate produced. Consequently the timing principle can be applied by observing how long it takes the pH in a given buffer solution to rise to a definite point. Such application has been made by Hunter and Dauphinee (19) to the measurement of arginase activity.

In the present procedure a buffer solution is used which sets the initial mixture at a pH of 6.7, and the time is measured which is required for the pH to rise to 7.7.

Reagents—

0.1 per cent phenol red.

10 per cent solution of urease to be tested.

1 M NaH_2PO_4, 13.8 gm. of $NaH_2PO_4 \cdot H_2O$ per 100 cc.

1 M K_2HPO_4, 17.4 gm. per 100 cc.

Buffered urea solution of pH 6.7 is prepared by dissolving 3 gm. of urea in 10 cc. of molar NaH_2PO_4, 10 cc. of molar K_2HPO_4, and sufficient water to make the volume 100 cc.

Control buffer of pH 7.7. Into a 100 cc. graduated cylinder measure 1 cc. of molar NaH_2PO_4 and 7 cc. of molar K_2HPO_4 and dilute to 80 cc.

Procedure

To 5 cc. of buffered urea solution of pH 6.7 in a 20 × 150 mm. test-tube, and to 5 cc. of control buffer of pH 7.7, without urea in a similar tube, are added 2 drops of phenol red. The solutions are brought to room temperature or to the temperature of a water bath. The temperature is recorded. 0.5 cc. of 10 per cent urease is added to each tube, and mixed with a footed rod, and a stop-watch is started. The exact time is noted that is required for the tube with the urea + enzyme to reach the same red shade as the control. The matching of colors is best made in a good light with a white background immediately behind the adjacent tubes. 50 mg. of urease (0.5 cc. of 10 per cent solution) containing 0.20 unit per mg. (an average preparation of acetone-precipitated jack bean urease) require 5 minutes at 20°.

TABLE II

Factors for Colorimetric Timing Method

Temperature	Factor	Temperature	Factor
°C.		°C.	
5	2.10	25	0.758
		26	0.727
10	1.62	27	0.689
11	1.54	28	0.653
12	1.47	29	0.620
13	1.40		
14	1.33	30	0.590
		31	0.562
15	1.26	32	0.536
16	1.20	33	0.510
17	1.14	34	0.485
18	1.08		
19	1.03	35	0.461
20	0.982	40	0.363
21	0.937		
22	0.890	45	0.304
23	0.842		
24	0.800	50	0.270
		55	0.245

If the colors match in less than 2 minutes, the determination is repeated with urease solution formed by diluting 1 volume of the 10 per cent solution to V volumes with water. Ordinarily no dilution is needed, and $V = 1$.

Calculation—

$$\text{(10)} \qquad \textit{Sumner units per mg.} = \frac{\text{factor} \times V}{\textit{time in min.}}$$

The factors are given in Table II. They were determined empirically by observing the time intervals required at different temperatures for urease of known activity to raise the pH to 7.7.

EXPERIMENTAL. MANOMETRIC

Effects of Too Much Urease or Too Little Protein on Activity Measurements—The activities of two preparations of urease as indicated by the technique outlined in the original method of Van Slyke and Cullen (1) were compared with the values obtained by (*a*) the manometric method, Procedure A, outlined above, and (*b*) the same manometric procedure modified by omitting the albumin. The results are recorded in Table III.

The lower "apparent units" by the titration method are due to the fact that more units of urease were present than this method was designed to measure, and so much ammonia was formed that the phosphate buffer did not suffice to hold the pH from rising above 7.2. When the manometric procedure was modified by omission of the albumin, results were also low, chiefly from the effect of the mercury. The correct results are given in the last column of Table III.

TABLE III

Diminution of Apparent Urease Activity by (a) Using So Much Urease That Final pH Exceeds 7.3 (Titrimetric) or (b) Omitting Egg Albumin (Manometric)

Preparation of urease, Squibb's double strength	Titrimetric; volume 6 cc , urease 16 7 mg per cc , urea 50 mg per cc , phosphate 0 417 M, digestion period, 15 min			Manometric; volume 6.5 cc., urease 0 154 mg. per cc , urea 23 mg per cc., phosphate 0 05 M,* digestion period, 5 min			
	No albumin			No albumin		5 per cent albumin	
	NH_3-N formed	Apparent Sumner units	pH at end of reaction	P_{CO_2}, a = 2 cc.	Apparent Sumner units	P_{CO_2}, a = 2 cc	Sumner units
	mg.	*per mg.*		*mm*	*per mg.*	*mm.*	*per mg.*
I	45.16	0.1505	8.7	47 2 (25.9°)	0.123	102.7 (25.5°)	0.272
II	48.49	0.1616	8.9	51.1 (26.0°)	0.132	115.5 (25.0°)	0.314

* In the manometric activity determinations the pH remained within the range 6.8 to 7 2 throughout the reaction period.

Changes in pH and Reaction Velocity When High Activity Urease Is Used in the Van Slyke-Cullen Activity Determination—In Fig. 1 the pH and velocity are plotted against both time and the amounts of ammonia nitrogen formed. The results were obtained with a 10 per cent solution of "double strength" urease (Preparation I, Table III) acting under the conditions of the Van Slyke-Cullen assay (1). The pH of the digestion mixture was observed at minute intervals in a glass electrode for 15 minutes at 20°. The ammonia liberated was measured in separate reaction mixtures by aeration and titration, as described by Van Slyke and Cullen (1, 2).

Effects of Presence of Metallic Mercury, and of Addition of Egg Albumin, When the Concentration of Jack Bean Protein Is Small—The results of three series of experiments are shown in Fig. 2. The digestions and analyses in two of the series were carried out in the manometric chamber, in the presence of metallic mercury, as in Procedure A. In the third series, the results of which are given by the intermediate curve, the digestions were carried out in a flask, without contact with mercury, by the technique of manometric Procedure B, except that no albumin was present. The incubations were conducted at room temperature and lasted 5 minutes. The values of P_{CO_2} observed were multiplied by the C factors in Table I, to give the amounts of ammonia that would be formed at 20°. The variable in each series was the concentration of "double strength" urease in the enzyme solution added.

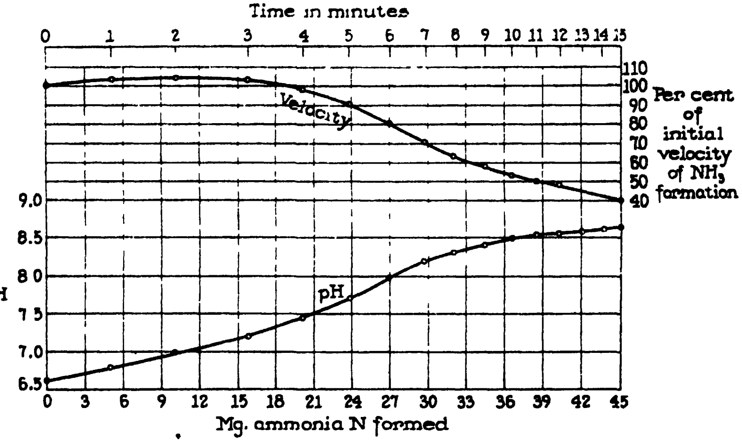

FIG. 1. Change of pH and velocity of reaction when 1 cc. of 10 per cent urease (27 Sumner units) acts at 20° on 5 cc. of molar urea in 0.5 M phosphate.

From the uppermost curve it is seen that under the conditions of Procedure A, with albumin added, the amount of ammonia formed is directly proportional to the amount of urease present.

The lowest curve, from the digestions in the presence of metallic mercury with no added egg albumin, was made with a urease preparation slightly stronger than that used for the other two curves; this lowest curve indicates that in the digests with the smallest amounts of urease nearly all of the latter was inactivated. When the concentration of urease powder exceeded 0.01 mg. per cc. of digest, the increase in rate of ammonia formation for each *further* increase in urease concentration paralleled the increase indicated by the uppermost curve. The explanation appears to be that when a solution containing less than 0.01 mg. of urease preparation per cc. was used the concentration of jack bean proteins, which constitute the greater part of the commercial "urease" employed, was not sufficient to protect the enzyme from inactivation. The inactivation was partly the effect of the mercury, and partly (middle curve, Fig. 2) the effect either of other substances in the reagents, or of mere dilution in the absence of protecting protein (10). That inactivation of the more dilute urease solutions occurred, even in the absence of mercury, is shown by the intermediate curve of Fig. 2.

In the Van Slyke-Cullen (1) activity estimation the use of 1 cc. of 10 per cent solution of crude soy bean urease provided sufficient protective protein to prevent such inactivation. When the smaller amounts required of the more active jack bean urease are used, especially the minute amounts used in the sensitive manometric procedures, addition of other protective protein is necessary.

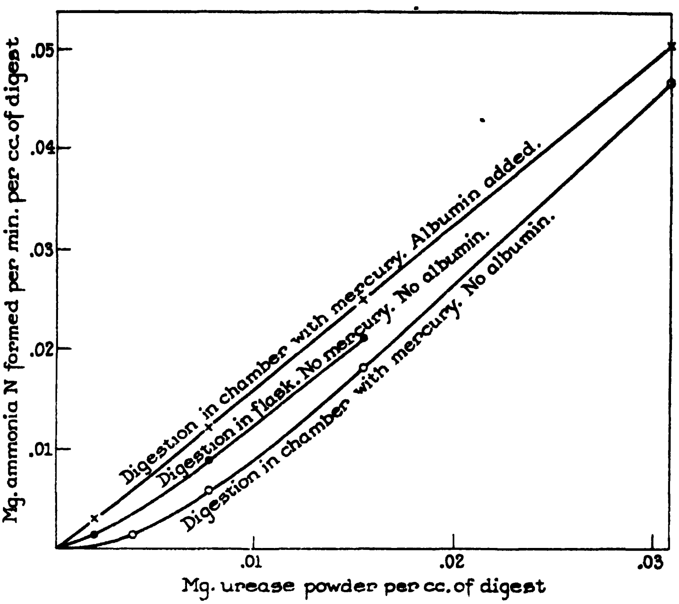

FIG. 2. Influence of albumin on activity of dilute solutions of urease in presence and absence of mercury.

Proportionality between Duration of Urease Action and Amount of Ammonia Formed under Conditions of Manometric Procedure A—Fig. 3 shows the degree of proportionality existing between the duration of the reaction and the amount of ammonia formed by 1 cc. of 0.1 per cent urease in the manometric chamber.

Temperature Coefficient of Urease Action under Conditions of the Activity

Measurements—With the manometric Procedure A reactions were run at different temperatures by regulating the temperature of the water jacket surrounding the Van Slyke-Neill blood gas chamber. Water of the desired temperatures was circulated through the water jacket before and during each incubation. The buffered substrate was allowed to come to temperature equilibrium within the chamber before addition of the enzyme. The enzyme solution was measured in a stop-cock pipette at room temperature. The pipette and its contents were then brought to a temperature approximating that of the substrate. After completion of the incubation and before extraction of the CO_2 the temperature was adjusted, when necessary, to fall within the range of Van Slyke's manometric factors ((4) p. 713).

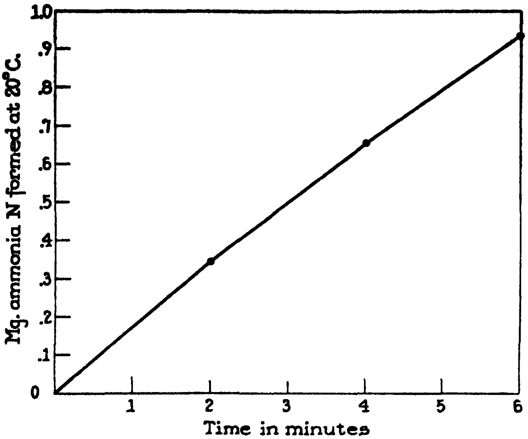

FIG. 3. Proportionality between amount of substrate hydrolyzed and duration of incubation under conditions outlined in Procedure A.

$$\frac{\text{Rate at } (t^\circ + 10^\circ)}{\text{Rate at } t^\circ} = \text{coefficient as recorded in Table IV}$$

$$\frac{\text{Rate at } 20^\circ}{\text{Rate at } t^\circ} = \text{factor } C \text{ for } t^\circ \text{ as recorded in Tables I and IV}$$

Table IV shows that the temperature coefficients for urease acting under the conditions of the present manometric method vary from 1.48 between 30–40° to 1.77 between 5–15°.

Under the conditions of the colorimetric method (Table II) the apparent temperature coefficient for the same temperature range varies from 1.62 to 1.67. The difference between the temperature coefficients obtained by the manometric method and the apparent coefficients calculated from data obtained by the colorimetric method is due partly to the effect of temperature on the pH of the buffers and consequently on the difference in the amount of $(NH_4)_2CO_3$ which is required to change the buffer from the initial pH to that at which the color in the two tubes match. In the colorimetric method the amount of ammonium carbonate required to bring about a matching is not the same at 5° as at 20° or 40°. Hence the true temperature coefficient can be determined only by the manometric or titrimetric methods. Correct activity values are given by the colorimetric method because corrections for all temperature effects, on buffer and indicator as well as on the enzyme, are incorporated in the factors of Table II.

TABLE IV

Temperature Coefficients of Urease As Determined by Manometric Procedure

Temperature	Factor *C*, Table I	Temperature coefficent
°*C.*		
5	2.31	
10	1.76	1.77
15	1.30	1.76
20	1.00	1.67
25	0.779	1.62
30	0.617	1.52
35	0.511	1.48
40	0.417	

SUMMARY

1. Egg albumin has been shown to protect urease in dilute solutions from inactivation.

2. The original manometric and titrimetric procedures for measuring urease activity have been modified for application to the more active urease preparations now available.

3. A simple colorimetric procedure has been added; the speed with which pH is raised in a specified phosphate-urea solution serves as a measure of the rate of ammonium carbonate formation, and of urease activity.

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THE ENZYMATIC DETERMINATION OF GLUTAMINE

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(Received for publication, May 16, 1944)

Existing methods of measuring glutamine depend upon either the liberation of ammonia by mild acid hydrolysis (1) or on the fact that when heated at 100° in neutral or slightly acid solution transformation to pyrrolidonecarboxylic acid occurs with disappearance of the amino nitrogen (2) determined by the nitrous acid method (3) and disappearance of the $—CH(NH_2)\cdot COOH$ group determined by evolution of its CO_2 when heated with ninhydrin (4, 5).

In view of the growing interest in the physiological rôle of glutamine (5–9) it seems desirable to have available also a specific enzymatic micromethod.

Krebs (9) demonstrated that the cortex of rabbit, pig, guinea pig, and sheep kidney contained an enzyme, glutaminase, which hydrolyzed glutamine to glutamic acid and ammonia, and the writer has described a preparation adapted to analytical use (10). The present paper outlines a method which uses this enzyme to determine the glutamine content of blood, plasma, and plant extracts and to assay the purity of glutamine preparations. Use of this method to assist in identifying glutamine amide nitrogen as a source of urinary ammonia has been published in a preliminary note (11).

Two procedures will be described. In one, the "filtrate nesslerization procedure," the digest is deproteinized and the ammonia is determined in the filtrate. In the other, the "distillation procedure," the ammonia is distilled *in vacuo* and nesslerized in the distillate. The filtrate method, though less accurate and less satisfactory than the distillation method, is included since it permits approximate estimation of glutamine when the distillation apparatus is not available.

Apparatus

Incubator or water bath at 38°.

Apparatus for micro ammonia determination (12).

Reagents Used Only in Filtrate Nesslerization Procedure

10 per cent sodium tungstate.

0.5 N H_2SO_4.

2.5 per cent potassium persulfate. Stored in ice box, fresh weekly.

1.0 per cent potassium gluconate. Stored in ice box, fresh weekly.

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Reagents Used Only in Distillation Procedure

Saturated borate buffer to give pH 10.0. A mixture of 47 cc. of 18 N NaOH (or 34 gm. of NaOH or 47.6 gm. of KOH), 185 gm. of borax (U. S. P. powder), and 1800 cc. of water is boiled for 15 minutes to remove traces of ammonia. The solution is cooled to about 30° and made up to 2 liters with water. 2 cc. of the diluted solution with 0.5 cc. of 1 M phosphate buffer of pH 7.2 give a solution of pH 9.9 (glass electrode).

0.04 N H_2SO_4 (approximate). 0.56 concentrated H_2SO_4 diluted to 500 cc.

Antifoam. 1 volume of sorbitan monolaurate[1] and 3 volumes of liquid paraffin.[2]

Reagents Used for Both Procedures

Potassium cyanide, 0.04 M, pH 7.2. To 2350 cc. of distilled water add 6.5 gm. of KCN and 150 cc. of 1 M NaH_2PO_4. The cyanide solution should be stored in the ice box and prepared fresh at least every week to keep the ammonia blank near its minimum.

Phosphate buffer, 1 M. To 1 volume of M NaH_2PO_4 are added 3 volumes of M K_2HPO_4; 0.5 cc. of this solution on dilution to 2.0 cc. gives pH 7.2.

0.0005 M $(NH_4)_2SO_4$ for Standard. 0.0140 mg. of NH_3-N per cc.

Nessler's solution, as prepared by Vanselow (13). 45.5 gm. of mercuric iodide together with 34.9 gm. of potassium iodide are dissolved in 150 cc. of water. 112 gm. of potassium hydroxide are added and the volume adjusted to 1 liter with water.

Glutaminase. The "25 per cent" emulsion of dialyzed kidney extract described in the accompanying paper (10). It should have an activity of at least 10 glutaminase units per cc.

PROCEDURE A. DIRECT NESSLERIZATION OF DIGEST FILTRATE

Collection of Plasma for Glutamine Determination—In the determination of plasma glutamine levels blood was drawn in oiled syringes to minimize hemolysis, and was mixed with 1 mg. of potassium oxalate, or 0.05 mg. of heparin, per cc. in CO_2-filled centrifuge tubes. The blood was centrifuged at once, in a cold room or ice box, and the separated plasma was recentrifuged to remove the few remaining cells. As shown by Conway (14), if whole blood is kept in an atmosphere of CO_2 until the centrifugation is complete, the ammonia content of the plasma is thereby reduced to a negligible amount. However, as ammonia blanks are run on the plasma in the present analysis, maintenance of the CO_2 saturation in the present analysis is not essential.

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[1] This is supplied by the Atlas Powder Company, Wilmington, Delaware, under the trade name of Span 20.

[2] For some purposes the mineral oil can be replaced to advantage by the same proportions of 2-methyl-2,4-pentanediol, obtained from the Commercial Solvents Corporation, 17 East 42nd Street, New York. A warm mixture of 1 part of glycerol monolaurate S (Glyco Products Company) in 4 parts of mineral oil is also a good antifoam.

Digestion of Sample with Glutaminase—To a sample of 1 cc. of plasma, or of other fresh neutral material containing 0.05 to 1.2 mg. of glutamine, 0.5 cc. of the phosphate buffer (to give a final pH of 7.3) then 0.5 cc. of 25 per cent suspension of dialyzed kidney are added and mixed. The mixture is incubated 1 hour at 38°.

Blanks—Simultaneously three blanks are run.

On the *reagents* other than the enzyme preparation, a blank is prepared in which the sample and enzyme are replaced by 1 cc. of water and 0.5 cc. of cyanide solution, respectively. The NH_3-N in the reagent blank is B_R.

On the *enzyme*, a blank is prepared in which 1 cc. of water replaces the sample, the enzyme and other reagents being present as in the analysis. The mixture is incubated in the same manner as the analyzed sample. The ammonia formed is the sum of that preformed in the enzyme preparation and reagents plus that formed from materials in the enzyme preparation during the incubation. The NH_3-N in the enzyme blank is B_E.

On the *sample*, a blank is prepared in which the sample is mixed with buffer plus 0.5 cc. of the cyanide solution instead of plus 0.5 cc. of enzyme suspension. The mixture is incubated in the same manner as the analyzed sample. In this blank one determines the ammonia preformed in the plasma or other material forming the sample, together with ammonia formed during the incubation by actions other than that of glutaminase. Included in the ammonia measured in this blank is approximately 5 per cent of the amide nitrogen of whatever glutamine is present in the sample. This ammonia is split from the amide group of the glutamine in three successive stages of the manipulation as follows: (*a*) during the 1 hour incubation 2 per cent of the amide nitrogen is liberated by spontaneous decomposition at that temperature; (*b*) during the period of 1 hour at room temperature (25°) between precipitation of the proteins and nesslerization of the filtrate another 2 per cent is liberated; (*c*) during the 5 minute period between addition of the Nessler's solution and the photometer reading 1 per cent is liberated by the action of the Nessler's reagent. The NH_3-N in the sample blank is B_S.

Deproteinization—Immediately at the end of the 1 hour incubation period 1 cc. of 10 per cent sodium tungstate solution is added to each digest and mixed. Then 2 cc. of the 0.5 N sulfuric acid are added and mixed. The solution is made up to a volume of 10 cc., mixed, and filtered.

Standard Transmittance Curve—Solutions containing 0 (S_0), 0.0140, 0.0280, and 0.0420 mg. of ammonia nitrogen per 5 cc. are prepared as follows: Of 0.5 mM $(NH_4)_2SO_4$ solution, portions of 0, 1, 2, and 3 cc. respectively are mixed with 0.5 cc. portions of a cyanide-phosphate solution which is prepared by mixing equal volumes of 0.04 M KCN[3] and the 1 M phosphate buffer. Water is added to bring each ammonia-phosphate-cyanide solution to exactly 5 cc.

Nesslerization—Shortly before nesslerization a solution is prepared by mixing equal volumes of the 2.5 per cent potassium persulfate solution and of the 1 per cent gluconate. To 5 cc. of each tungstic acid filtrate and to each standard add 1 cc. of this persulfate-gluconate solution, which serves to prevent development of turbidity from reduction of the mercury in the Nessler's reagent (15). After the material is mixed, 0.5 cc. of Nessler's reagent is added and mixed.

Measurement of Transmittance—During the interval between 4 and 6 minutes after the Nessler's solution is added the transmittance of the solution is measured in a photometer, light of a wave-length of 450 mμ and a cuvette which provides a transmitting layer of about 1 cm. length being used. The transmittance of the standard S_0 is taken as 100 per cent. When an instrument with a transmittance scale which extends above 100 per cent is used, it is convenient after adjusting the instrument to read 100 per cent with S_0, to record the transmittance in the absence of the cuvette, and when compensating for any instrumental fluctuations during the series to adjust to this reading without the cuvette rather than to read 100 per cent transmittance with S_0.

Special Timing Conditions for Blank B_S—In order to make the ammonia, split by non-specific reactions from glutamine in the sample blank, total the estimated 5 per cent of the glutamine amide nitrogen, the period during which the tungstic acid filtrate stands before nesslerization is set at 1 hour, before the Nessler's solution is added, and the period of 5 minutes between adding the Nessler's solution and measuring the transmittance must be accurate within ±1 minute.

Calculation—The glutamine amide nitrogen (half the total glutamine nitrogen) in the sample analyzed is calculated as

$$G = 1.05(N - B_E - B_S + B_R) \tag{1}$$

The amount of glutamine is $G \times 10.43$.

G = glutamine amide nitrogen
N = the total ammonia nitrogen measured in the incubated digest
B_E = " NH_3-N found in the enzyme blank
B_S = " " " " " sample "
B_R = " " " " " reagent "

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[3] As it decreases the amount of color developed with Nessler's reagent, cyanide must be added to each standard in a concentration equal to that present in the unknown.

Derivation of Equation 1—Besides the symbols used in Equation 1 the following are employed.

s_1 = NH_3-N preformed in the sample
s_2 = " formed in the sample blank from spontaneous decomposition of non-glutamine substance of the sample during incubation at 38°
e_1 = NH_3-N preformed in the enzyme suspension
e_2 = " formed from substances of the enzyme suspension during incubation at 38°
r_1 = NH_3-N in reagents (buffer, etc.) other than the enzyme suspension

From the conditions described

$$N = G + s_1 + s_2 + e_1 + e_2 + r_1 \tag{2}$$
$$B_S = 0.05G + s_1 + s_2 + r_1 \tag{3}$$
$$B_E = e_1 + e_2 + r_1 \tag{4}$$
$$B_R = r_1 \tag{5}$$

Subtraction of the sum of the blanks, equations (3) + (4) + (5) from (2) gives

$$N - (B_S + B_E + B_R) = 0.95G - 2r_1 \tag{6}$$

Since $r_1 = B_R$, we can substitute B_R for r_1. Making this substitution and solving for G we have

$$G = 1.052(N - B_S - B_E + B_R) \tag{7}$$

PROCEDURE B. NESSLERIZATION OF DISTILLATE FROM DIGEST

2 cc. of plasma or 1 to 5 cc. of other neutral solution (containing 0.05 to 0.6 mg. of glutamine), 0.5 cc. of molar phosphate buffer, and 0.5 cc. of 25 per cent suspension of dialyzed kidney in 0.04 M KCN are incubated 1 hour at 38°. Appropriate blanks for ammonia production in the enzyme (B_E), in the sample (B_S), and in the reagents (B_R) are prepared as indicated for Procedure A and incubated at the same time. The tubes containing unknowns and blanks are then placed in ice water or in a thermos bottle partly filled with dry ice. The contents of each tube are transferred in turn to a 50 cc. thick walled centrifuge tube with a minimum of wash water, and 1 drop of antifoam mixture is added.[4] Alkalinization with borate and vacuum distillation of the ammonia into 5 cc. of 0.04 N H_2SO_4 are carried out in the apparatus described by Archibald (12), with the bath at 50°

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[4] Caprylic alcohol cannot be used as antifoam reagent without special precautions, because it coagulates the red colloidal ammonia-mercury compound formed in Nessler's solution. However, if 1 cc. of persulfate-gluconate (15) mixture is added before the Nessler's solution the use of 4 drops of caprylic alcohol as antifoam is permissible. The sorbitan laurate has the advantage over caprylic alcohol that it does not distil over to any appreciable extent, 1 drop lasts for the whole determination, and the later use of gluconate-persulfate is unnecessary.

and the solution at 35–42°. After addition of 2 cc. of the borate to the enzyme digest, the pH of the mixture is 9.9.

The distillation of the ammonia is complete in 5 minutes. The distillate and rinsings are run directly into a 10 cc. volumetric flask and the volume is adjusted to 10 cc.

Standards are made up in 10 cc. volumetric flasks with 1, 2, 3, or 4 cc. of 0.0005 M $(NH_4)_2SO_4$, 5 cc. of 0.04 N H_2SO_4, and water to volume.[5] The Nessler blank is made up, 5 cc. of 0.04 N H_2SO_4 and water to 10 cc.

To each flask (Nessler's blank, other blanks, standards, and samples) is then added 0.5 cc. of Nessler's reagent. The resulting solutions have been read in our analyses in Coleman's Spectrophotometer 10-S with a 5 mμ slit at 450 mμ wave-length against the Nessler blank which is set at 100 per cent transmission. Thus correction is automatic for the small amount of ammonia present in the 5 cc. of 0.04 N acid, and the distilled water used to make up to volume. Correction for the ammonia present in the water used to transfer the enzyme digest to the distilling tube is included in B_R.

Calculation—The calculation is by Equation 8, which is the same as Equation 1, except that the constant 1.05 is replaced by 1.022.

$$G = 1.022(N - B_E - B_S + B_R) \tag{8}$$

The derivation of Equation 8 is similar to that of Equation 1. However, the correction factor, 1.022, replaces the factor 1.05 of Equation 1 because the procedure used here, when applied to determination of the blank B_S, liberates NH_3 from only 2.2 per cent, instead of 5 per cent, of the amide group of the glutamine present. The freeing of 2 per cent of the amide nitrogen as ammonia which occurs in Procedure A during the hour of standing of the tungstic acid filtrate prior to nesslerization is here avoided, and, in place of 1 per cent freed by decomposition of glutamine in B_S by Nessler's solution in Procedure A, only 0.2 per cent in Procedure B is liberated during the distillation.

DISCUSSION OF TECHNIQUE

Conditions for Nesslerization of Filtrate in Procedure A—Direct nesslerization of filtrates from the enzyme digest gives satisfactory results only when the concentration of interfering substances is small. With uremic blood or tissue filtrates there is present too high a concentration of urea, which shifts, and changes the shape of the absorption curve, and of creatinine, which reduces Nessler's reagent and is largely responsible for the turbidity so frequently encountered in direct nesslerization. The gluconate-persulfate mixture recommended by Gentzkow (15) is adequate to prevent clouding only when the concentration of creatinine is less than 5 mg. per

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[5] There is no need in this procedure to add cyanide to the standards, since none is retained by the acid distillates.

100 cc. Gentzkow obtained theoretical recovery of ammonia on direct nesslerization of urease-treated blood filtrates only when λ = 490 to 510 mμ. We find that the wave-length at which recovery of added ammonia is theoretical is increased in the presence of pyruvate and decreased by the presence of urea and amino acids. In the presence of the latter, recovery of added ammonia is theoretical at about 450 mμ. With our reagents and technique of direct nesslerization recovery of ammonia added to dog tissue or plasma filtrates has been theoretical only at 450 to 470 mμ. Since Nessler's reagent is more sensitive at 450 than 500, and absorption by the Nessler blank is increased only slightly by using the shorter wave-length, we have used λ = 450 mμ for measuring the ammonia in the "direct" nesslerization procedure as well as after distillation.

Relative Advantages of Nesslerizing Distillate Rather Than Filtrate (Procedure B Versus Procedure A)—The distillation procedure has the following advantages: As no protein precipitation is necessary, there is no loss of glutamine in a bulky precipitate. The whole of the enzyme digest instead of an aliquot is available for analysis, and smaller concentrations of glutamine can be determined. No creatinine, amino acids, or other filtrate materials are present to complicate conditions for nesslerization of the distillate. No gluconate-persulfate is necessary. Any cyanide distilled off is not caught by the acid, hence cyanide need not be added to the standards. Absence of interfering constituents permits selection of a wave-length over a wide range. With the distillation technique, results are more accurately reproducible, and blank values are more consistent, than with the direct nesslerization of filtrates. This is especially true of the correction value for preformed ammonia. The ammonia liberated from glutamine itself, in blank determination B_S, during the distillation at 38–42° and pH 9.9 in 5 minutes is only 0.2 per cent of the glutamine amide N, whereas in Procedure A the blank B_S includes about 2 per cent of the glutamine amide nitrogen which is freed as ammonia during the clearing procedure, and an additional 1 per cent liberated by the action of the Nessler reagent. While correction for ammonia from these sources is made in the blank in Procedure A, the correction is less constant and accurate than the smaller one obtained in the distillation technique.

Results

Table I compares the results by the two methods of the enzymatic determination of amide N of glutamine preformed in plasma, of glutamine added to plasma, and of pure glutamine in distilled water.

Fig. 1 indicates the rate of action of the enzyme on pure glutamine solution and on plasma and shows that the reaction is usually complete within ½ hour.

Tables II and III show the glutamine amide N concentration in a number of plasmas from fasting human subjects and from fasting dogs. The parallel results "by ninhydrin" are by a method which will be described in a later publication by Dr. P. B. Hamilton.

Table IV indicates the concentration of glutamine (as determined by the enzymatic method) in synovial fluid and yolk and white of fresh chicken egg. Table V shows the purity of glutamine preparations as indicated by the enzymatic and ninhydrin methods.

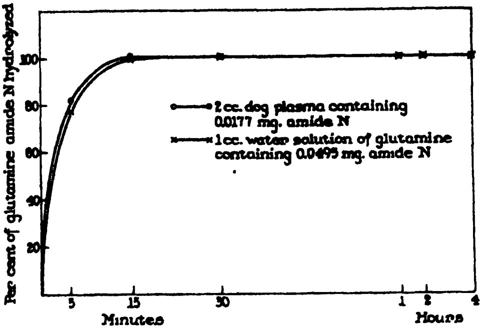

FIG. 1. Time curve of hydrolysis of glutamine in water solution and in plasma at pH 7.2 at 38° by 0.5 cc. of 25 per cent suspension of dog kidney in 0.04 M KCN.

TABLE I

Recovery of 0.4 Mg. of Glutamine by Two Methods

	Glutamine amide N in dog plasma	Recovery of glutamine added to plasma	Recovery of glutamine added to water
	mg per 100 cc.	*per cent*	*per cent*
Nesslerization after distillation	0.62	100.0	100.0
	0.62	100.2	100.2
Direct nesslerization of protein-free filtrates	0.65	95.2	106.4
	0.66	95.2	103.7

Enzymatic analysis for glutamine in urine is at present not entirely satisfactory, presumably because inhibiting substances are present in urine. Recovery experiments, however, indicate that the amide N of free glutamine in urine is almost insignificant in amount.

In dog urine excreted between the plasma samplings indicated in Table III under Dog B-18, June 24, glutamine amide N was less than 0.024 mg. per 100 cc.; the excretion rate was less than 0.01 mg. per minute.

Urine values for dogs in acidosis are of the same order of magnitude. The urine output in a normal human is 11 mg. of amide N per 24 hours. This is of the same order of magnitude as the value for glutamic acid (4.3 mg.

per 100 cc.) reported by Cohen (16) as present in human urine. Cohen's method of determination would include glutamine as well as glutamic acid.

TABLE II

Glutamine Amide N Content of Plasma of Fasting Humans

Patient	Age	Sex	Diagnosis	Glutamine amide N		Total α-amino N	α-Amino N of glutamine as per cent of total α-amino N
				By glutaminase	By ninhydrin		
	yrs.			*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
R. A.	34	M.	Normal	0.87		3.96	22.0
D. S.	25	"	Convalescent, malaria	0.74			
				0.74			
G. L.	20	"	" "	0.82			
				0.82			
J. D.	22	"	" "	0.66		3.72	17.7
P. T.	22	"	" "	0.99			
				0.98			
R. K.	22	"	Malaria (in chill)	0.37		3.36	11.0
L. P.	22	"	Convalescent nephritic	0.80		4.79	16.7
W. G.	46	"	" "	0.70		3.80	18.4
J. W.	23	"	" "	0.66		4.04	16.3
R. B.	12	"	" "	0.72		3.43	21.0
C. T.	6	F.	" "	0.50		2.70	18.5
L. R.	5	M.	" "	0.66		3.37	19.6
W. M.	12	"	" "	0.66		3.78	17.5
S. R.	14	"	" "	0.75		3.27	23.0
L. W.	4	F.	Nephrotic, Oct. 26, 1942	0.30		2.94	10.2
			Nov. 2, 1942	0.20		2.66	7.5
			" 6, 1942	0.66			
J. A.	6	"	"	0.71			
M. R.	5	"	Convalescent nephrotic	0.87		4.52	19.2
C. S.	32	M.	" "	0.78		4.17	18.7
B. W.	14	F.	" "	0.85		3.32	25.6
C. M.	24	M.	Terminal nephritic	0.84		4.44	18.9
N. O.	20	"	" "	0.94		5.70	16.5
K. T.	44	"	" " coma, arterial	0.40	0.53		
			" " " jugular	0.38	0.43		
D S.	8	"	Advanced progressive muscular dystrophy	0.61		3.10	19.7
G. T.	9	"	" "	0.61		3.26	18.7
F. R.	56	F.	Chronic myelogenous leucemia	0.64		2.82	22.7

Discussion of Results

The production of ammonia in the enzyme blank, though reduced markedly by the presence of cyanide, is still appreciable. It is assumed that

TABLE III

Glutamine Amide N Content of Plasma of Fasting Dogs

Dog. No.	Date	Condition of dog	Glutamine amide N							
			By glutaminase				By ninhydrin			
			Sample No.	Arterial	Sample No	Renal venous	Sample No	Arterial	Sample No	Renal venous
				mg. per 100 cc.		*mg per 100 cc.*		*mg. per 100 cc.*		*mg. per 100 cc.*
B-10	Dec. 4, 1942	Acidosis	1	0.79	1	0.67				
			2	0.81	2	0.58				
			3	0.77	3	0.60				
	June 21, 1943	"	1	1.19	1	0.76	1	1.22		
			2	1.22	2	0.81	2	1.23		
			3	1.20	3	0.81	3	1.12		
B-18	Dec. 14, 1942	Alkalosis	1	0.80	1	0.80				
			2	0.87	2	0.84				
			3	0.88	3	0.83				
	June 24, 1943	"	1	1.05	1	0.89			1	0.79
			2	1.09	2	0.95			2	0.93
			3	1.21	3	0.97			3	1.07
B-15	Dec. 4, 1942	Acidosis	1	1.09	1	0.81				
				1.09		0.81				
			2	1.05	2	0.79				
				1.05		0.79				
			3	1.03	3	0.85				
				1.03		0.85				
	Oct. 5, 1943	"	1	0.82	1	0.65				
			2	0.73	2	0.64				
B-19	Dec. 10, 1942	"	1	0.68	1	0.46				
				0.68		0.47				
			2	0.69	2	0.44				
				0.69		0.48				
			3	0.69	3	0.47				
				0.68		0.46				
						Systemic venous				
C		Normal				1.04				
D		"				0.69				
E		"				0.87				
F	Feb. 8, 1942	"				1.00*				
G		"				0.97				

* This value was obtained by direct nesslerization.

the ammonia formed in the enzyme blank results from action of cathepsins and other peptide-splitting enzymes on the kidney proteins with liberation of glutamine from the proteins and subsequent hydrolysis by glutaminase. The observation that the enzyme blank increases much less on incubation when bromosulfalein is present favors the conclusion that the ammonia formed on incubating the enzyme blank is derived from glutamine and is liberated by the action of glutaminase. Bromosulfalein has been shown (10) to inhibit glutaminase. Under the conditions outlined for glutamine determination in plasma the concentration of protein in the enzyme preparations is so high that the addition of plasma proteins does not increase that portion of the blank which is due to liberation of ammonia from proteins. Consequently the method is applicable to plasma without correction for effects of plasma proteins. The upper curve in Fig. 1 shows

TABLE IV

Glutamine Amide N in Synovial Fluid and Egg White and Yolk

	Glutamine amide N
	mg. per 100 cc.
Human synovial fluid	1.28
White of unincubated egg, Sample 1	0.25
" " " " " 2	0.16
Yolk of unincubated egg	3.52

TABLE V

Determinations of Purity of Samples of Glutamine

Sample	NH_3-N preformed in sample	Amide N split by glutaminase	Amide N by ninhydrin
	per cent	*per cent of theoretical*	*per cent of theoretical*
Glutamine (rye grass exudate)*	0.05	100.24	100.0
" from beets*	0.1	99.0	
Commercial Sample L-2	1.32	93.00	
		93.88	94.0
" " L-7	0.72	81.17†	
		81.20	81.9
" " L-8	0.44	80.9	83.1
" " L-H-13	0.10	93.3	
" " L-149	0.05	85.7	
" " L	99.2	0.8	
" " S	8.0	73.0	
" " F	73.3	<3.0	<1.0

* Supplied through the kindness of Dr. Pucher and Dr. Vickery.

† NH_3-N liberated by heating 2 hours at 100° with 0.02 N H_2SO_4, 80.6 per cent of theory.

the difference between the ammonia in (*a*) the plasma plus kidney extract, (*b*) the blank containing kidney extract. This difference reaches a maximum. If ammonia liberated from glutamine derived from proteolysis in the kidney extract were less than when plasma proteins were also present, no such maximum would be attained.

The presence of an appreciable concentration of glutamine in chicken egg yolk and the maintenance of moderate concentrations during 6 days of incubation suggest an analogy between conditions in the egg and those existing in plant seeds. The localization of glutamine to the yolk is remarkably complete in the unincubated egg but decreases during incubation.

It can be seen from Table II that the plasma values for convalescent patients ranged from 0.5 to 1.0 mg. of glutamine amide N or from 5.7 to 10.4 mg. of glutamine per 100 cc. The lower limit of normal may be considered to be about 0.6 mg. of glutamine amide N or 6.2 mg. of glutamine per 100 cc. In fasting dogs the plasma level in arterial or systemic venous blood varied between 0.68 and 1.22 mg. of amide N per 100 cc. or between 7.1 and 12.7 mg. of glutamine per 100 cc. The level varied appreciably in the same dogs from one time to another.

The average of the values for arterial and systemic venous dog blood is 0.92 mg. of amide N, or 9.6 mg. of glutamine, per 100 cc. The average for the renal venous blood of dogs in acidosis was markedly lower; *viz.*, 0.67 mg. of glutamine amide N or 7.0 mg. of glutamine per 100 cc. It has previously been shown (11) that the kidneys remove amide nitrogen from glutamine to form ammonia excreted in the urine.

The finding, indicated in Fig. 1, that the velocity curve of ammonia production by glutaminase is almost identical, whether the enzyme acts on a glutamine solution or on plasma, affords added evidence that the substance measured in plasma is free glutamine.

Cohen (16) reported that the glutamic acid level in human blood plasma was 2.8 mg. per 100 gm. His method involved conversion of glutamic acid to succinic acid and subsequent measurement of O_2 uptake in the presence of succinoxidase. His method was, as he realized, not specific for glutamic acid, but measured glutamic acid plus 70 per cent of the glutamine plus 17 per cent of the glutathione present. The value reported by Cohen is equivalent to 4.0 mg. of glutamine per 100 cc. Comparison of the values found by Cohen for glutamine + glutamic acid concentration with our values (Table II) for glutamine in normal (convalescent) human plasma makes it appear probable that practically all of the material measured by Cohen as glutamic acid was in fact glutamine.

The total α-amino acid nitrogen of free amino acids in normal human plasma, measured by the ninhydrin-CO_2 method, has been found by Hamilton and Van Slyke (17) and by Cramer and Winnick (18) to average 4.1 mg.

per 100 cc. The median normal (convalescent) glutamine amide nitrogen of 0.8 mg. per 100 cc. indicates that glutamine α-amino nitrogen constitutes about 20 per cent of the total α-amino nitrogen of the plasma, and that the total glutamine nitrogen equals 40 per cent of the α-amino nitrogen.

The fact that so much of the α-amino nitrogen circulating in mammalian plasma is in the form of glutamine points to this compound as an important one in intermediary metabolism of nitrogen. That it is the chief source of urinary ammonia in dogs has been demonstrated (10).

Spontaneous hydrolysis of the labile amide nitrogen of glutamine accounts for a part of the "γ" ammonia which Conway observed (14) was formed in plasma *in vitro*. In this connection it is interesting to note that Conway observed that the amount of phosphate liberated on incubation of rabbit plasma was insufficient to account for the NH_3-N liberated if this were derived from adenosine after dephosphorylation of vegetable adenylic acid. He observed in human plasma *in vitro*, at room temperature, a production of 0.14 mg. of NH_3-N per 100 cc. in 1 day.

SUMMARY

An enzymatic method for the determination of glutamine is described. The method is specific for glutamine in the absence of purine nucleoside derivatives bearing NH_2 groups. Evidence is presented which indicates that the concentration of interfering substances in dog and human plasmas is negligible and that the method can be used to determine the concentration of glutamine in plasma.

Two analytical procedures are described. In the "filtrate" procedure the proteins are removed from the digest and the ammonia in the filtrate is determined with Nessler's solution. In the "distillation" procedure the ammonia is distilled at 38–42° *in vacuo* and the distillate is nesslerized. The distillation method is the more accurate.

The glutamine values obtained by the enzymatic method indicate a range of glutamine amide nitrogen content from 0.6 to 1.0 mg. per 100 cc. or from 6 to 10 mg. of glutamine per 100 cc. of normal human plasma. Values down to 0.2 mg. were obtained in pathological plasma. In dogs the values in systemic plasma ranged from 7 to 13 mg. of glutamine per 100 cc. Glutamine was measured also in synovial fluid, and dialysates of chicken egg whites and yolks.

The author wishes to acknowledge his debt to Dr. D. D. Van Slyke for many helpful suggestions, to Mr. J. A. Plazin for technical assistance, to Dr. P. B. Hamilton for some of the ninhydrin analyses recorded, and to Dr. R. A. Phillips for help in procuring dog blood and kidneys.

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PREPARATION AND ASSAY OF GLUTAMINASE FOR GLUTAMINE DETERMINATIONS

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(Received for publication, May 16, 1944)

Krebs (1) demonstrated that the cortex of kidneys of the sheep, guinea pig, pig, and rabbit contained an enzyme, glutaminase, which hydrolyzed glutamine to ammonia and glutamic acid. The present paper describes the preparation and standardization of kidney glutaminase in a form which makes the enzyme readily applicable for microestimation of glutamine in blood and various other biological materials.

I. PREPARATION OF GLUTAMINASE EMULSION

Procedure

Dog kidneys provide a glutaminase preparation which is more active and specific than beef kidneys. Beef kidneys nevertheless yield a preparation which gives satisfactory results[1] and can be used if dog kidneys are not available.

The kidneys are stripped free of perirenal fat and capsules and emulsified with 3 parts of an ice-cold solution of 0.04 M KCN which has been adjusted to pH 7.2 by addition of NaH_2PO_4. With amounts of tissue of the order of 100 gm. the emulsification is most conveniently performed by placing the tissue and solution in a Waring blendor and "blending" for 5 minutes. An alternative procedure preferable with small amounts (5 to 25 gm.) is to grind the tissue and solution in a mortar with sand. The fluid part of the mixture is strained through a sac of unbleached muslin (fine mesh) to remove fibrous connective tissue. Emulsification and straining are done in an ice box or a room at 0°. The resulting emulsion is sufficiently homogeneous and if kept at 0° retains adequate activity for 24 hours. In order to conduct emulsification, filtration, and dialysis at or near 0°, the kidney, cyanide solution, and apparatus including the blendor or mortar are cooled to 0° before use.

The portion of emulsion to be used at once is placed in a Visking cellulose[2] sausage casing (No. 27/32 Nojax) and is shaken vigorously for 2 hours at 0° in a vessel containing 2.5 liters of 0.04 M KCN (6.5 gm. of KCN) which has had its pH adjusted to 7.2 by the addition of 150 cc. of molar NaH_2PO_4.

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[1] The enzyme is present in normal human kidneys but it is either present in smaller amounts or absent in kidneys of patients who have died of chronic nephritis or nephrosis, or who have had arteriosclerotic disease involving the kidneys. The concentration of enzyme in a number of human kidneys will be reported in detail elsewhere.

[2] Supplied by the Visking Corporation, 6735 West 65th Street, Chicago, Illinois.

Notes on the Preparation

Whole dog kidneys stored frozen in solid CO_2 retain their activity for more than a year. Stored at 3° they retain adequate activity for several days. The extract, however, loses an appreciable part of its activity daily when stored either at 3° or in the frozen state. Hence when small amounts of enzyme of maximum activity are required for analyses at intervals of days or weeks, it is advisable to keep as stock source of the enzyme a dog kidney frozen fresh and preserved in solid CO_2. Small amounts, 5 to 10 gm., are broken off with a hammer as needed and extracted as described above. For these amounts the mortar and pestle are better suited than the Waring blendor.

The blank for preformed ammonia in this enzyme preparation increases more slowly in the presence of cyanide than in its absence. Nevertheless ammonia is produced even in the dialyzed, cyanide-treated kidney preparations at ice box temperature, and more rapidly at room temperature or 38°. Hence to obtain minimum blank values, dialysis of the enzyme preparation should be stopped not more than 20 minutes before the start of the incubation.

A high percentage of the glutaminase in kidney adheres tenaciously to cellular components insoluble in ordinary extractives. This is especially true of beef kidney; 0.8 per cent NaCl solution is the best extractive so far found. In a single instance, seven times repeated freezing (chilling to −70°) and thawing rendered the enzyme almost completely extractable from insoluble débris, but attempts to repeat this under conditions which appeared identical have been fruitless.

Attempts have been made to concentrate the enzyme by isoelectric precipitation, fractional precipitation with acetone at 0°, and with safranine, but as yet, in the most active fractions, the ratio of enzyme to protein has not been increased appreciably.

Consequently attention has been devoted to obtaining an enzyme reagent which was specific for glutamine, and was sufficiently active to complete the hydrolysis of glutamine in a short time. To this end means were sought to activate the glutaminase, and to inhibit other enzyme systems present which were capable either of liberating ammonia from sources other than the amide group of glutamine, or of causing the disappearance of ammonia liberated by action of the glutaminase.

Blanks are diminished by removal of diffusible extracts by means of dialysis and by addition of cyanide. Cyanide serves also to augment the activity of the glutaminase and to depress the liberation of ammonia from asparagine and from α-amino groups. By the use of pH 7.2 for action of the glutaminase, approximately maximum activity is obtained.

A commercial enzyme mixture, Polidase S,[3] may for some purposes provide a convenient preparation of glutaminase. It is a stable, powdered enzyme mixture which contains both glutaminase and adenosine deaminase, but is free from asparaginase. When freed of ammonium ion by dialysis against 0.04 M KCN, it is a suitable preparation to use for glutamine determination in the presence of relatively high concentrations of asparagine. Owing to the high proteinase, phosphatase, and adenosine deaminase content, it is not suited to analysis for free glutamine in material containing proteins, adenosine, or phosphate derivatives of adenosine.

Because certain plants synthesize glutamine, Vickery *et al.* (2) have postulated that the roots of these plants contain glutaminase. An enzyme capable of liberating ammonia from glutamine even in the presence of 0.01 M KCN has been demonstrated in the roots of plants which store fixed nitrogen as free glutamine. The concentration of this glutaminase is increased when the soil in which the plants are grown is dressed with ammonium salt (data to be reported later). Work is under way which it is hoped may lead to the preparation of glutaminase free from enzymes which deaminate purine nucleosides and their derivatives, so that the enzymatic determination of glutamine will not be limited to plasma and other nucleoside-free material.

II. ASSAY OF GLUTAMINASE ACTIVITY

Use is made of the principle that the time required for action by an enzyme on a given amount of substrate is inversely proportional to the amount of active enzyme present (3). The amount of product formed is constant at the moment of the significant measurement; hence the effect of products as a variable is cancelled. The following procedure is designed to use minimal amounts of the expensive glutamine.

Apparatus

Water bath 38°.

Spectrophotometer or colorimeter.

Apparatus for microdetermination of ammonia previously described (4).

***Reagents*—**

The *molar phosphate buffer, pH 7.2, borate buffer, pH 10.0, 0.04 N H_2SO_4, antifoam 0.04 M KCN* adjusted to pH 7.2, *Nessler's reagent*, and *standard ammonium sulfate solution* as described in the accompanying paper (5).

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[3] The Polidase S is prepared by the Schwarz Laboratories, Inc., 202 East 44th Street, New York.

Glutamine, 0.0025 M solution. Weigh out between 3.4 and 4.0 mg. of glutamine. Dissolve this in a volume of water such that there are 10 cc. of solution for 3.654 mg. of glutamine. The cc. of water used will be 2.74 times the number of mg. of glutamine weighed.

Bromosulfalein,[4] *0.1 per cent solution,* or *quinone, 0.01 per cent aqueous solution.* Quinone distils over with the ammonia, and if used in larger quantities than recommended interferes with the nesslerization. Bromosulfalein, however, may be used in higher concentrations if desired.

Assay Procedure

The 25 per cent dialyzed emulsion obtained by the procedure described above is diluted with 4 volumes of the 0.04 M KCN solution of pH 7.2.

Into each of seven test-tubes measure, in the following order, 0.5 cc. of the molar phosphate of pH 7.2, 1.0 cc. of the 2.5 mM glutamine solution, and 0.5 cc. of the 5 per cent glutaminase solution. All solutions are brought to 38° before they are added, and the tubes are kept at 38°. At intervals of 2, 5, 10, 15, 20, and 30 minutes the action of the enzyme in the respective tubes is stopped by addition of 1 cc. of either the bromosulfalein or quinone solution. A blank is included in which the glutamine solution is replaced by water. The blank is incubated for 15 minutes. As soon as the enzyme action in each tube is stopped, the tube is stoppered and immersed in ice water, and kept there until the ammonia is distilled. If it is necessary or convenient to store the tubes for more than 2 hours before distillation, they should be placed in solid CO_2 instead of in ice water. The chilling is to retard spontaneous evolution of ammonia from the glutamine left unchanged by the enzyme and the catalytic breakdown of amide groups of protein by the bromosulfalein. The contents of each tube are washed with 2 cc. of water into the micro ammonia apparatus (4), 2 cc. of borate buffer are added, and the ammonia is distilled, nesslerized, and measured against standards containing 0 to 0.07 mg. of NH_3-N as described in the accompanying paper ((5) Procedure B).

Calculation of Glutaminase Units

The results are plotted on coordinate paper, mg. of NH_3-N as ordinates and minutes as abscissa. By interpolation, the number of minutes is ascertained which are required to form 0.01 mg. of ammonia nitrogen. The units of glutaminase present in the 0.5 cc. sample of 5 per cent kidney emulsion are calculated as

$$\text{Units glutaminase} = \frac{10}{\text{min. to form 0.01 mg. } NH_3\text{-N}}$$

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[4] Supplied as a 5 per cent aqueous solution by Hynson, Westcott and Dunning, Inc., Baltimore, Maryland.

An example is given in Fig. 1. In the case of this enzyme preparation 7.0 minutes were required to form 0.01 mg. of NH_3-N. The 0.5 cc. of preparation therefore contained 10/7 = 1.43 units of glutaminase.

The unit as defined above is the amount of glutaminase that will liberate 0.01 mg. of NH_3-N from glutamine in 10 minutes under the conditions prescribed for the determination.

The glutaminase per cc. of undiluted 25 per cent emulsion is 10 times the amount found by the above procedure in 0.5 cc. of the 5 per cent emulsion.

A slightly less accurate but simpler procedure requiring only a single incubation period can be followed when only approximate values for activity are required or when only weak preparations of glutaminase are available for assay. The sample and blank are made up and incubated as outlined above for 15 minutes. From Fig. 1 read the point on the abscissa corresponding to the number of mg. of amide N split in 15 minutes. Assuming that there is inappreciable loss of enzyme activity during the 15 minutes, the time reading on the abscissa will be directly proportional to the enzyme activity. For example, if 0.005 mg. of amide N is split in 15 minutes, the corresponding point on the abscissa of Fig. 1 for a preparation containing 2.86 units per cc. reads 3.4 minutes. The strength of the tested sample is therefore 2.86 × 3.4/15 = 0.65 unit per cc. For very active preparations shorter incubation periods should be used.

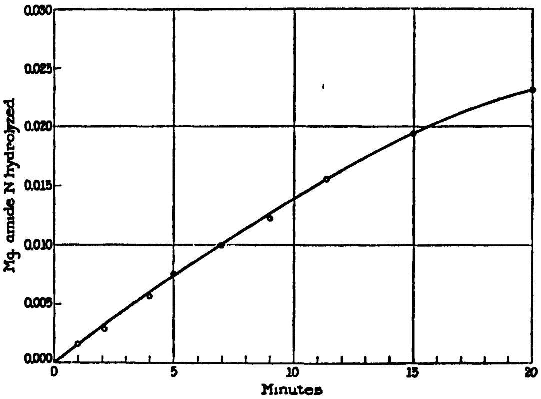

FIG. 1. Graph of ammonia formation in assay of glutaminase

III. EFFECT OF PH ON ACTIVITY OF GLUTAMINASE

Mixtures were prepared as described for assay of the glutaminase, except that, by addition of varying amounts of molar NaH_2PO_4 or K_2HPO_4 solution, the pH was varied between 5.3 and 8.6. The pH determinations of the mixtures were made in a glass electrode with unincubated mixtures made up in the same way. All digestions were run for 10 minutes. Blank corrections for the amount of ammonia liberated at these pH values from glutamine and from the kidney extract were obtained from separate curves prepared in the same way except that in one case cyanide replaced kidney extract, and in the other water replaced glutamine.

A similar series was run, with the difference that KCN was omitted from the mixtures. The results are given in Fig. 2. They indicate maximal activity at pH 7.5 and a plateau from pH 7.0 to 8.3 over which activity exceeds 90 per cent of the maximal. Below pH 6.0 activity is zero. The results agree, in general, with those obtained by Krebs (1) with sheep kidney and guinea pig liver glutaminase acting in the absence of cyanide. Data obtained from several different preparations suggest, as do those reported in Fig. 2 and by Krebs (1) with sheep kidney, that there are two kidney glutaminases with different pH optima.

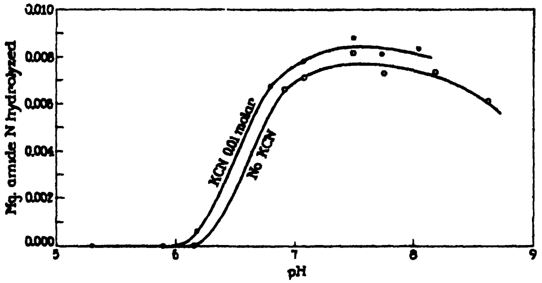

FIG. 2. Effect of pH on activity of glutaminase

It will be noted from Fig. 2 that the addition of KCN increased, only slightly, the activity of the enzyme. In this case the enzyme was prepared from kidney which had been stored in solid CO_2 for 4 months.

IV. SPECIFICITY OF KIDNEY GLUTAMINASE AND ITS ENHANCEMENT BY KCN

Despite the fact that Krebs (6) reports that ground kidney preparations contain no *l*-amino acid deaminase, some of our fresh glutaminase preparations, in the absence of cyanide, produced ammonia from α-NH_2 groups of *l*-amino acids, including glutamine. Besides this ability to produce ammonia from α-NH_2 groups, some fresh preparations of kidney (especially of beef kidney) liberated large amounts of ammonia from asparagine, presumably from the amide group through the action of asparaginase.

Whatever the source of the ammonia from asparagine, the addition of KCN to a concentration of 0.0025 M in the digestion mixture decreased the ammonia formation from asparagine to 35 per cent of the rate in the absence of KCN in the case of dog kidney, and to 65 per cent in the case of beef kidney. This same concentration of cyanide decreased the liberation of ammonia from "amigen" (casein hydrolysate) to 15 per cent of the rate in the absence of KCN in the case of fresh beef kidney preparations and to 5 per cent in the case of fresh dog kidney preparations.

On the other hand, the glutamine-hydrolase activity of fresh kidney is either enhanced or stabilized, or both, by the presence of 0.0025 M KCN. In one instance the ammonia liberated from 0.0025 M solution of glutamine in 1 hour was increased 2-fold with fresh beef kidney and 7-fold with fresh dog kidney preparations by the presence of 0.0025 M KCN. The presence of this concentration of cyanide reduces the action of kidney extracts on α-NH_2 groups to insignificant levels. Ammonia formation in appreciable amounts is then restricted (in the case of aliphatic compounds) to the amide group of glutamine. Under the conditions for glutamine determination outlined in the accompanying paper (5) no appreciable amount of ammonia is liberated by the extract of dog kidney from asparagine, amino acid mixtures (casein hydrolysate), or glutamic acid, proline, lysine, ornithine, arginine, urea, glycocyamine, creatinine, creatine, guanidine, guanine, adenine, uracil, cytosine, thymine, nucleic acids (yeast or thymus), yeast adenylic[5] or muscle adenylic acids,[5] vicine, inosine, uric acid, allantoin, glucosamine, glutathione, thiamine, nicotinamide, or nicotinamide nucleoside.[6] The only ammonia-yielding substrates[7] other than glutamine so far

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[5] The slow but continuous liberation of small amounts of ammonia when kidney suspension acts upon either muscle or yeast adenylic acids and the similarity of rate of ammonia production in the two cases suggest that the kidney phosphatase acts on the adenylic acids to liberate adenosine (a reaction which is retarded by the cyanide and the high concentration of phosphate present (7)) and that the adenosine is then deaminated.

[6] Dr. Schlenk (8) kindly supplied us with samples of codehydrogenase I and nicotinamide nucleoside. The latter preparation (70 to 80 per cent pure) liberated small amounts of ammonia in the presence of the enzyme preparation. However, this ammonia was believed to arise from some of the precursor, codehydrogenase I or muscle adenylic acid in the nucleoside preparation, since prolongation of the incubation period resulted in a marked decrease in the rate of ammonia formation. Alternatively, this fall in rate could be due to the presence in the kidney extract of an enzyme which hydrolyzed the nicotinamide from the carbohydrate and thereby diminished the substrate available for deamination.

[7] Krebs reported (1) that phenacetylglutamine and benzoylglutamine are not attacked by kidney extract. He states that glutaminylglycine and glutaminylglutamic acid yielded no ammonia in the presence of rat brain glutaminase, but that isoglutamine was hydrolyzed slowly.

In the presence of the glutaminase preparation the only available sample of *dl*-citrulline in our hands liberated small amounts of ammonia at a slow but almost constant rate. When 10 mg. of citrulline were present in 2 cc. of digestion mixture under the conditions of analysis outlined above, 2.4 per cent of one nitrogen was split off as ammonia in 2 hours, and 6 per cent in 8 hours. Even if the rate of this hydrolysis were doubled when the substrate was purely a levo isomer, inappreciable amounts encountered are codehydrogenase I[6] and the two purine nucleosides bearing NH_2 groups; *viz.*, adenosine and guanosine. Vicine, which is a nucleoside of the pyrimidine, divicine, and glucose, is not deaminated, although it bears two amino groups. The fact that cyanide extract of kidney, used as glutaminase, liberates ammonia from amino purine nucleosides and from codehydrogenase I does not disqualify the extract as a reagent for determining glutamine in plasma, because the concentrations of adenosine or its derivatives in plasma are very small compared to the concentration of glutamine. Spectrophotometric transmittance measurements with light of wave-length between 230 and 300 mμ on dialysates of dog and human plasma obtained by the technique of Hamilton and Archibald (9) indicate a maximum possible total concentration of adenosine, adenylic acids, adenosine triphosphate and diphosphate, and codehydrogenase I of less than 4×10^{-5} M or the equivalent of less than 0.06 mg. of ammonia N per 100 cc. of plasma. Dr. Herman Kalckar has studied the decrease in this

TABLE I

Action of 0.5 Cc. of 25 Per Cent Suspension of Dog Kidney (Dialyzed in 0.04 M KCN) + 0.5 Cc. of Molar Phosphate of pH 7.2 + 1.0 Cc. of Substrate

Substrate	Amount of substrate incubated	NH₃-N liberated as per cent of one N of substrate			
		½ hr.	1 hr.	2 hrs.	4 hrs.
	mg.				
Glutamine	0.7633	100	100	100	100
Asparagine	1.359		2.5		
Muscle adenylic acid	1.319		10.5	18.0	
Yeast " "	1.325		8.2	11.4	
Adenosine	0.6937	99	100	100	
Guanosine	0.6711	4.1	8.2	21.4	53.7
Nicotinamide nucleoside 75% pure	0.49			4.8	
Codehydrogenase I 85% pure	0.611	8.9	14.8	43.4	
Amigen (casein hydrolysate)	1.788		0.52*		

* Per cent of total nitrogen.

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of ammonia (less than 0.01 mg. per 100 cc. of plasma) would be set free from the low concentrations of citrulline (0.3 to 1.5 mg.) normally present in plasma (Archibald, unpublished).

The observation, however, is of interest in view of the similarity of structure of glutamine and citrulline; the CH_2 adjacent to the acid amide group in glutamine is replaced by $—CH_2·CH_2·NH—$ in citrulline. As with glutamine, the enzymatic liberation of ammonia from citrulline is slower if the 0.01 M KCN is omitted from the digest. In one instance the rate of liberation of ammonia from citrulline was 25 per cent less when KCN was omitted. The action of preparations of glutaminase on citrulline is of interest in connection with the disputed rôle of glutamine in urea synthesis. The hydrolysis of citrulline by preparations of liver glutaminase is also being investigated.

absorption on treatment of dog plasma filtrates with phosphatase plus adenosine deaminase and concludes that there is less than 0.3 mg. of adenylic acid per 100 cc. of plasma. If *all* of this were deaminated by the enzyme, the ammonia resulting therefrom would be less than 1.5 per cent of the amide N found in plasmas. Furthermore, there is sufficient adenosine deaminase in plasma (6) (especially in slightly hemolyzed plasma) to destroy adenosine were it present, whereas we have been unable to find glutaminase in plasma.

The relative rates of liberation of ammonia nitrogen from several nitrogenous substances are given in Table I. In the experiments tabulated the technique described for assay of glutaminase activity was employed, with the listed substrates substituted for glutamine.

TABLE II

Action of 2 Cc. of Heparinized Dog Plasma on 1 Cc. of Substrate in Presence of 0.5 Cc. of Molar PO_4, pH 7.2, and 0.5 Cc. of 0.04 M KCN

Substrate	Amount of substrate incubated	NH_3-N liberated in 1 hr. at 38°	NH_3 liberated as per cent of one N of substrate per hr.
	mg.	*mg.*	*per cent*
Glutamine	0.7633	0.0001	0
Asparagine	1.359	0.0001	0
Muscle adenylic acid	1.319	0.0015	2.9
Yeast " "	1.325	0.0013	2.5
Adenosine	0.6937	0.0054	14.9
Guanosine	0.6711	0.0016	5.4
Amigen (casein hydrolysate)	1.788	0.0000	0
Plasma without substrate		0.0015*	

* Not corrected for the 0.0004 mg. of NH_3-N resulting from spontaneous breakdown of glutamine.

Table II shows the effect of incubating several of these substances with plasma under the conditions outlined for the use of the kidney preparation for the analysis of glutamine in plasma. The absence of glutaminase, asparaginase, and α-amino acid deaminase from plasma is apparent. The presence of nucleoside deaminase and of either phosphatase or nucleotide deaminase is apparent.

V. INHIBITORS OF GLUTAMINASE

The effect of various inhibitors on the action of glutaminase (15 minutes at 38° at pH 7.2) was observed in a mixture made up of the following constituents: 0.5 cc. of 5 per cent dialyzed suspension of kidney in 0.04 M KCN of pH 7.2; 0.5 cc. of molar phosphate, pH 7.2; 1 cc. of 0.0025 M glutamine;

1 cc. of inhibitor solution. In the case of the control analysis, 1 cc. of water replaced the inhibitor solution. The concentration of inhibitors and percentage decreases in enzyme activity observed at pH 7.2 are recorded in Table III.

The author wishes to thank Dr. L. Michaelis and Dr. S. Granick for assistance in determining the ultraviolet transmittance curves of plasma dialysates.

TABLE III

Inhibition of Glutaminase Activity at pH 7.2

Inhibitor	Concentration of inhibitor in digestion mixture		Inhibition of glutaminase
	per cent	*M*	*per cent*
p-Benzoquinone	0.0013	0.00014	73
	0.00013	0.000014	12
Bromosulfalein (disodium sulfonate of phenoltetrabromophthalein)	0.17	0.002	100
	0.017	0.0002	90
	0.0017	0.00002	14
Atabrine (3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)-butylaminoacridine)	0.33	0.0093	100
	0.033	0.00093	60
Synthetic vitamin K (2-methyl-1,4-naphthohydroquinone 3-sodium sulfonate)	0.10	0.0036	0

SUMMARY

A procedure is described for the preparation from kidneys of a glutaminase extract which liberates ammonia quantitatively from the acid amide group of glutamine and which is sufficiently active and specific for use as an analytical reagent to determine glutamine in blood plasma.

Addition of potassium cyanide has been found to enhance the activity of the glutaminase and to retard the liberation of ammonia from asparagine and the action of α-deaminase.

The optimum pH of the dog kidney glutaminase is at 7.5. From pH 7.0 to 8.3 the activity exceeds 90 per cent of the maximal. The enzyme's action is entirely inhibited at pH below 6.

A procedure for assaying glutaminase activity is described, based on the inverse relation between activity of enzyme and time required to decompose a given amount of substrate.

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AN APPARATUS FOR THE DETERMINATION OF THE GASEOUS METABOLISM OF SURVIVING TISSUES IN VITRO AT HIGH PRESSURES OF OXYGEN*

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(Received for publication, May 10, 1944)

The authors have recently reviewed (1) a relatively old problem, oxygen poisoning, which was first discovered by Paul Bert in 1875, but which has received only scattered attention until the present war brought it once more into prominence. Experimental studies of the biochemistry of the phenomenon required a method whereby the gaseous metabolism of isolated surviving tissues or their separate enzymatic components could be studied under pressures of oxygen greater than 1 atmosphere. The present paper describes what has proved to be a successful apparatus and technique for this purpose. Because the problem has not, we believe, been extensively investigated from this point of view (with the exceptions mentioned below), the apparatus and method involve a number of new and unique problems, and are therefore presented in considerable detail. Actual experimental results will be reserved for subsequent papers. The basis of the technique is the familiar Warburg method for the manometric study of gaseous metabolism, subject to the modifications which the special condition of high pressure makes necessary. We have been unable to find in the literature any descriptions of apparatus designed to accomplish the present purpose with the sole exception of that of Libbrecht and Massart (2). They constructed a pressure chamber to contain a manometric apparatus of the Warburg type. Small motors in the pressure chamber controlled from outside performed the necessary manipulations of the Warburg apparatus. To prevent burning out the motors, the chamber was filled with nitrogen at high pressure, but the respiratory vessels proper, being connected to small balloons containing oxygen, were filled at high pressure (5 atmospheres) upon elevation of the pressure in the chamber. No other details are furnished by the authors and apparently only one series of reported experiments appeared in the literature, those on the action of oxygen on succinoxidase. These have been discussed by the present authors (1).

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania. Permission for publication granted by the Office of Scientific Research and Development.

Pressure Chamber

The apparatus (Fig. 1) consists essentially of a cylindrical steel pressure chamber 32 inches in maximum diameter, 53 inches high (inside), and of approximately 60 liters capacity, enclosing six Warburg manometers and vessels so constructed as to allow the necessary manipulations and readings to be made from the outside, while constant temperature and pressure are maintained within.

Fig. 1 shows the main features of construction, and for purposes of estimation of dimensions, is drawn to scale. It is not proposed to give all detailed measurements and specifications in the present paper, since these are best worked out to meet individual requirements. The walls of the main shell are $\frac{3}{8}$ inch steel, with $\frac{1}{2}$ inch welded flanges and bottom, and a bolted, removable 1 inch top. Six evenly spaced vertical windows of $\frac{3}{4}$ inch Plexiglas provide easy reading of the manometers, and a seventh similar window, midway between two of the others, is used to read the pressure gages and thermometer. Ample light is supplied by a 300 watt floodlight placed 18 inches above a 4 inch circular window of $\frac{3}{4}$ inch Plexiglas, in the top. In practice, the depression made by mounting this circular window inside the top is filled with water in order to prevent local heating of the plastic by radiation from the lamp, with consequent loss of tensile strength.

A number of openings for the entrance of movable shafts from the outside are provided, and are made gas- or water-tight as the case may be. The upper two stop-cock controls (*11* and *12* in Fig. 1) are made gas-tight by single composition stuffing boxes known as Garlock "closures." This type of seal makes motion of the control shafts reasonably easy when lubricated with a water suspension of graphite. In order to avoid accidental shifting of the stop-cocks while the manometers are being shaken, these upper controls are disengaged by sliding outward when not in use. The pressure outward on these is approximately 45 pounds at 8 atmospheres, and so it was found necessary to lock them in place by means of a pivoted bar. The lower controls (used to compress the rubber tubing at the lower end of the manometers), *13* in Fig. 1, together with the shaft transmitting power to the shaking mechanism and that used to provide stirring, are all under water, and are made pressure-tight by a graphite packing shown in the case of the stirrer in Fig. 3. The composition closures were found to be adversely affected by water, so that they deteriorated rapidly.

Of available transparent plastics, Plexiglas was found to possess the requisite physical properties for the purpose. Based on data from the manufacturers, the calculated maximum stresses provide factors of safety of 10 on the vertical windows and 4 on the upper window, at 38° and 8 atmospheres absolute. The mounting is a matter of importance in safety and

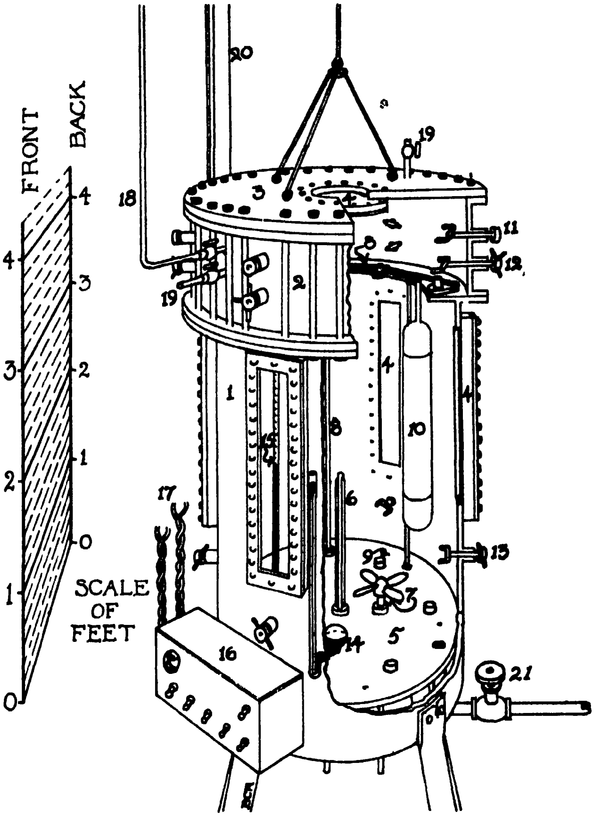

FIG. 1. Scale, perspective drawing of the high pressure chamber. *1*, main shell; *2*, upper section of shell; *3*, removable top; *4*, Plexiglas windows; *5*, bottom of shell; *6*, vertical immersion heater; *7*, stirring propeller; *8*, shaft, connecting bars, and pivoted holders for mounting and shaking the manometers; *9*, brass cups mounted in the bottom for the support of the lower ends of manometer frames; *10*, compensating vessel mounted in place. *11*, *12*, and *13*, manually rotated stop-cock and manometer fluid level controls; *14*, thermoregulator; *15*, part of one manometer seen in place through its window; *16*, control box for all electrical circuits; *17*, A.C. and D.C. power supplies; *18*, oxygen inlet pipe; *19*, auxiliary inlet and outlet pipes for flushing, etc.; *20*, swivel davit supporting wire rope hoist over ball bearing sheaves to worm-gear hand winch behind apparatus (not shown); *21*, water connection for filling and emptying chamber. The water level is just below the flange supporting the shaking apparatus, *8*.

durability, and consists in all cases of ¼ inch steel retaining plates held by ¼ inch bolts, the Plexiglas being drilled for full clearance and sealed with $\frac{1}{16}$ inch rubber gaskets. To prevent crazing and maintain clear visibility, the Plexiglas surfaces are washed with soap and water and finished with wax at weekly intervals.

Other essential features of the construction are apparent from Fig. 1 or are treated in detail below. Not shown in the diagram is a subsidiary connection of the water inlet to a hydraulic pressure tester. To minimize the possibility of blowouts in work with the gas under pressure, the apparatus is tested weekly. For this purpose, all glass apparatus is removed and the chamber filled completely with water at 115 to 125 pounds pressure for 15 minutes.

Temperature Maintenance

A semipictorial diagram of the heating unit is given in Fig. 2. Heat is supplied by a long, double element, vertical immersion heater (H_1 and H_2), with a maximum capacity of 600 watts. S_1 is a mercury contact relay, normally closed. Its coil is activated through a sensitive D.C. relay, S_2 (normally open), through the thermoregulator, *T. R.* In the normal position, the heaters are connected in parallel through a variable resistor, R_1. When temperature is reached, S_2 closes, opening S_1 and reducing the heat to that supplied by H_1 alone. The maximum and minimum heat requirements were established by measuring the radiation of the apparatus at average room temperature and allowing variation either side. This arrangement was found preferable to a simple, all or none circuit with fixed heat input because of the lag encountered in the thermoregulator. The variable R_1 is used to vary the total heat input. This was found to be a fairly critical adjustment, since the rate of radiation varies as the fourth power of the room temperature.

The thermoregulator (*T. R.*, Fig. 2) depends upon the relation between vapor tension of acetone and temperature. Acetone was chosen because of common, easily obtained substances, with boiling points below 38°; it shows the greatest change in vapor tension per °C. at 38°, amounting to 14 mm. of Hg. Ether, with a change of vapor pressure of about 29 mm. per °C., has the disadvantage that it requires a superimposed pressure of 130 mm. of Hg above atmospheric to maintain it in liquid state. The acetone, floated on mercury in a thick walled spherical bulb of about 50 cc. capacity within the chamber, transmits its pressure to a column of mercury outside of 1 mm. diameter (*M. C.*, Fig. 2). The large ratio of volume between bulb and column renders the volume change negligible, allowing maximum sensitivity. The shape and wall thickness of the bulb reduce transmission of the high pressure in the chamber to a negligible minimum. The lag thus occasioned does not decrease appreciably the accuracy of regulation. The mercury column is outside the pressure chamber and is easily adjustable.

At 8 atmospheres, the thin walled bulb of the standard thermometer is sufficiently compressed to give a positive error of 0.5°. This difficulty was solved by the use of a sealed mercury jacket, as shown to the left in Fig. 2 (*T. M.*). The thick wall of the surrounding tube prevents transmission of the pressure through the mercury jacket, and the air space above prevents its transmission by distortion of the rubber stopper. The increase in lag resulting from this arrangement does not interfere with accurate adjustment.

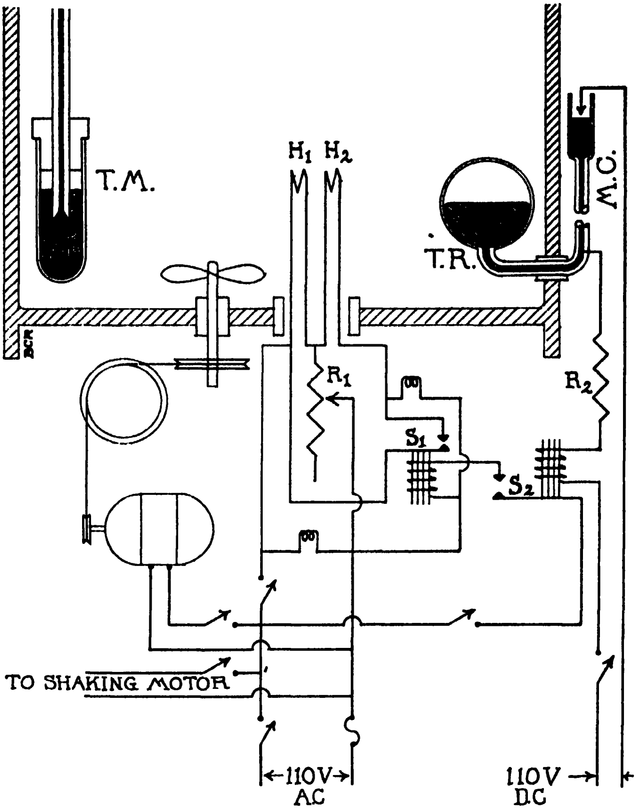

FIG. 2. Semipictorial diagram of the heating unit wiring, thermoregulator, and thermometer assembly. The parts are not drawn to scale. For the symbols used, see the text.

At first sight, the problem of stirring would not seem of great consequence but in practice it became complicated. A vertical shaft in the bottom of the tank was dictated by reasons of space and efficiency. A simple belt drive twisted through 90° to a horizontal motor developed mechanical difficulties. A direct coupled, vertical centrifuge motor developed insoluble lubrication difficulties in the packing box owing to the high speed necessary,

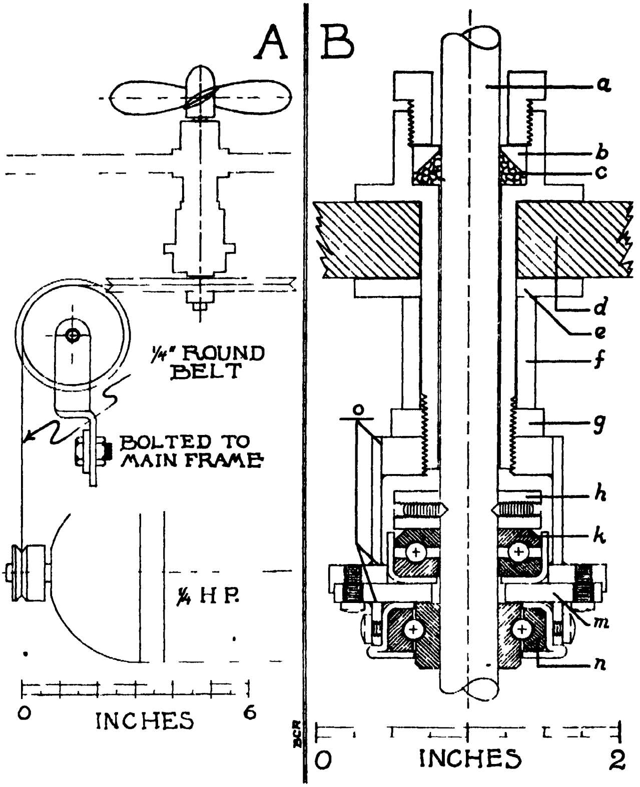

Fig. 3 *A*, assembly of stirring unit; *B*, enlarged, working drawing of the combination stuffing box and bearing. *a*, ⅜ inch brass shaft; *b*, brass collar, driven down on *c*, graphite packing; *d*, bottom of main shell; *e*, steel washer; *f*, steel shim; *g*, hex nut; *h*, brass thrust collar, held by two opposing safety set-screws, countersunk in shaft; *k*, thrust bearing Boston gear (No. 602); *m*, steel thrust plate, held by three screws; *n*, radial bearing Boston gear (No. 5368), held by two set-screws placed to avoid interference with the screws of *m*; *o*, silver-soldered joints. The unlabeled parts of the frame are steel.

causing ultimate overloading of the motor, and a proper gear reduction unit was impossible to obtain. The final design has proved so satisfactory that because of the problems met in its development it is considered worth presenting in detail.

Fig. 3 shows the construction of the stirrer transmission, and should be largely self-explanatory. Section *A* shows the whole assembly, while section *B* gives the constructional details of the combination bearing and stuffing box. To obtain quiet operation, the ¼ horse power, single phase A.C. driving motor is mounted on steel legs with rubber washers between the motor and legs and between the legs and the floor. In addition, ¼ inch leather belting was cemented with a 2 inch lap to give a continuous belt. The flat, ⅛ inch steel strip used to mount the idlers afforded sufficient spring to maintain proper belt tension. Leakage is held to an occasional drop by graphite packing. The bearing is thoroughly lubricated every 20 to 30 hours of operation with light grease. The four-bladed propeller is 4¾ inches in diameter, with a 5½ inch pitch, driving upward at 500 R.P.M. The resultant water circulation is well above the minimum required. Both shaft and propeller are built of brass.

As indicated in Fig. 1, the chamber is filled to about five-sixths capacity with tap water through a valve-equipped inlet in the bottom. The volume of oxygen under pressure is hence relatively small, thus diminishing the explosive force in case of an accidental blowout of any part of the apparatus. All the Warburg apparatus except stop-cocks and certain connections is submerged, thus making for easy maintenance of constant temperature, within ±0.02°.

Pressure Maintenance

Oxygen is supplied from a standard 220 cu. ft. cylinder through a manually controlled reduction valve and ¼ inch copper tubing to the inlet valve (*18*) shown in Fig. 1. Measurement of absolute pressure must be accurate to 0.02 atmosphere in 8 for the proper measurement of gas changes in the Warburg vessels. (This will be apparent from the discussion of the gasometric method below.) It was found that standard Bourdon type pressure gages are subject to errors in excess of 5 per cent, which prompted development of a simple, accurate gage, shown in Fig. 4, *A*.

The construction should be apparent from the figure. Aside from the use of a reasonably accurate 1 cc. pipette of about 2 mm. internal diameter, most conveniently graduated by 0.01 cc., none of the dimensions is critical. The top of the pipette is cut off 4 to 5 mm. above the zero mark and flared slightly to receive a small plug of rubber which is driven in exactly to the zero mark. The bottom is cut off 2 to 3 cm. below the 1 cc. mark and inserted in a 2-hole rubber stopper which in turn fits into a short section of test-tube. The internal diameter of this short section of a test-tube is so large relative to that of the pipette that a considerable rise of mercury in the pipette makes an inappreciable difference of level in the test-tube (*b*, Fig. 4). The relation between the length of the markings on the pipette and the volumes as marked, *i.e.* mm. per cc., is determined.

The gage is filled with sufficient mercury (and a few drops of free water to maintain aqueous vapor tension) and its level adjusted before insertion of the plug to the zero mark.

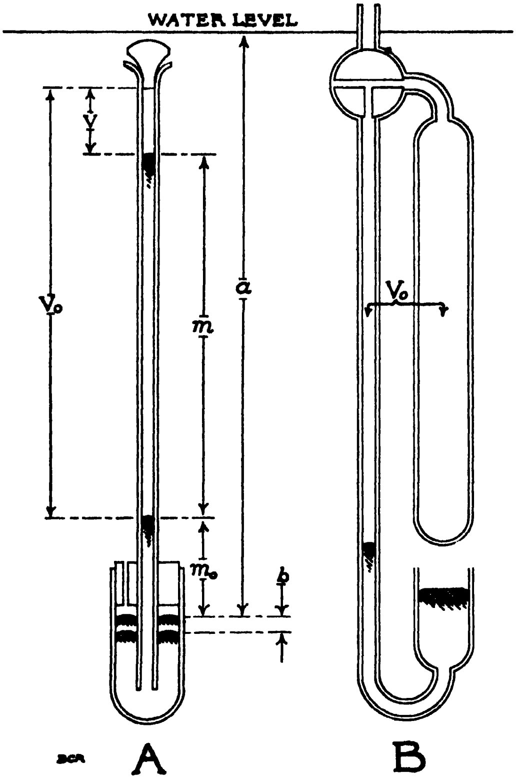

FIG. 4. *A*, absolute pressure gage; *B*, differential pressure gage (not drawn to scale). For the symbols used, see the text.

Upon introduction of gas the pressure in the chamber is calculated from the gage readings as follows (see Fig. 4, *A*):

Let P = total pressure in the chamber, in atmospheres; h_0 = initial hydrostatic pressure in the gage, in atmospheres. This is the sum of two factors: (1) the hydrostatic pressure of the water outside the gage; (2) the hydrostatic pressure of the mercury in the gage. These are shown as a and m_0 respectively in Fig. 4.

h = final hydrostatic pressure in the gage, in atmospheres. This is equal to h_0 plus two new factors: (1) A change in level of the mercury in the test-tube section with a corresponding increase in the water column a, labeled b

in Fig. 4. As has been pointed out above, this is negligible. (2) An increase in the height of the mercury in the gage pipette, shown as m in the diagram. This is clearly not negligible. But since the proportionality between the volume readings and the length of the column is easily measured, $m = k (V_0 - V)$, and $h = h_0 + k (V_0 - V)$.

w = aqueous tension, atmospheres
V_0 = volume of gas in the gage in cc., when P = 1 atmosphere
V = " " " " " " " " at any other pressure

Then, temperature being constant,

$$(1 + h_0 - w)V_0 = (P + h - w)V$$

Hence,

$$P = (1 + h_0 - w)\frac{V_0}{V} - h + w$$

If we introduce the proportionality between h, V_0, and V,

$$P = (1 + h_0 - w)\frac{V_0}{V} - h_0 - k(V_0 - V) + w \quad (1)$$

In practice, an important simplification is made by adjusting h_0 equal to 0 once and for all. To do this, the original level of the mercury in the pipette (m_0) is so adjusted before introduction of the rubber plug at the top that its hydrostatic pressure just balances that of the water column (a). The gage is calibrated as follows: The bath is filled to the proper level at the desired temperature (usually 38°) and left open to the atmosphere. P is then determined from a reading of the barometer, V is simultaneously read, and V_0 is calculated from Equation 1. It has been found convenient to construct a graph for other pressures up to 8 atmospheres, relating P to readings of V. Unless the rubber plug, and hence the setting of h_0, is disturbed, this graph serves for all time. The pipette can be easily read to an estimated 0.001 cc., giving calculated pressures to 0.01 atmosphere.

Differential Pressure Gage—The gas phase of the Warburg system is isolated from the circumambient atmosphere by closing the stop-cocks when the desired pressure is reached; the reasons for and the details of this arrangement are explained below. However, since stop-cocks blow *out* and not *in*, leakage at the stop-cocks, which was found otherwise to occur, was eliminated by maintaining pressure in the chamber at a slight differential pressure of 50 mm., Brodie's solution, above the pressure in the Warburg system. This is done by manual control of the reduction valve on the oxygen tank from time to time, as guided by the differential pressure gage shown in Fig. 4, *B*. As in the absolute gage, a 1 cc. pipette of about 2 mm. bore is used to measure changes in volume, but because the original mercury level (equivalent to the h_0 in the absolute gage) is not adjustable, and the column may fall as well as rise, the mercury reservoir is carried to the side as shown. In order to provide a high degree of sensitivity, the volume in the gage is increased by the auxiliary vessel (shown to the right of the pipette). The position of the 3-way stop-cock is dictated by the dimensions of the chamber and the available space for a stop-cock control passing to the outside, and is shown below the water line only for diagrammatic convenience. The stop-cock is left open to the circumambient gas until the desired pressure is reached, and is closed to the position shown on the diagram at the same time as the stop-cocks of the Warburg manometers.

The mathematical calculation of the differential pressure is the same as in the case of the first gage for the measurement of total pressure, except that 1 is replaced by P_0, the initial pressure in the chamber. However, since only approximate calculations are needed, second order values may be neglected and it is easy to show that the differential pressure is given by the equation,

$$\Delta P = \left(\frac{P_0}{V_0}\Delta V\right) - \Delta h$$

where

P_0 = initial pressure in atmospheres
ΔP = differential pressure, atmospheres
ΔV = change of volume, cc.
Δh = " " hydrostatic pressure of mercury in the gage, atmospheres

But, as before, there is a proportionality between Δh and the scale readings of the gage; *i.e.*, $\Delta h = k\Delta V$. Hence the above equation becomes

$$\Delta V = \frac{\Delta P}{\dfrac{P_0}{V_0} - k}$$

But k is of the order of 0.3 and P_0/V_0 never exceeds 8/100. Hence we may neglect the latter, and as a sufficient approximation we obtain

$$-\Delta V = \frac{\Delta P}{k} \tag{2}$$

Since ΔP is maintained at about 50 mm. of Brodie's solution ($= 50 \times 10^4$ atmospheres), ΔV is then about 0.015 cc., an easily readable difference of volume from the initial. Since by Equation 2 the readings of the differential pressure gage are for practical purposes independent of the total pressure, it may be calibrated, if desired, as follows: A Warburg manometer with vessel is placed in the apparatus, the arm not connected with the vessel being left open to the ambient pressure at some convenient level, *e.g.* 8 atmospheres. The pressure is then raised or lowered until there is a change in level of the manometer fluid of 50 or 100 mm., and the corresponding change in the differential pressure gage is noted.

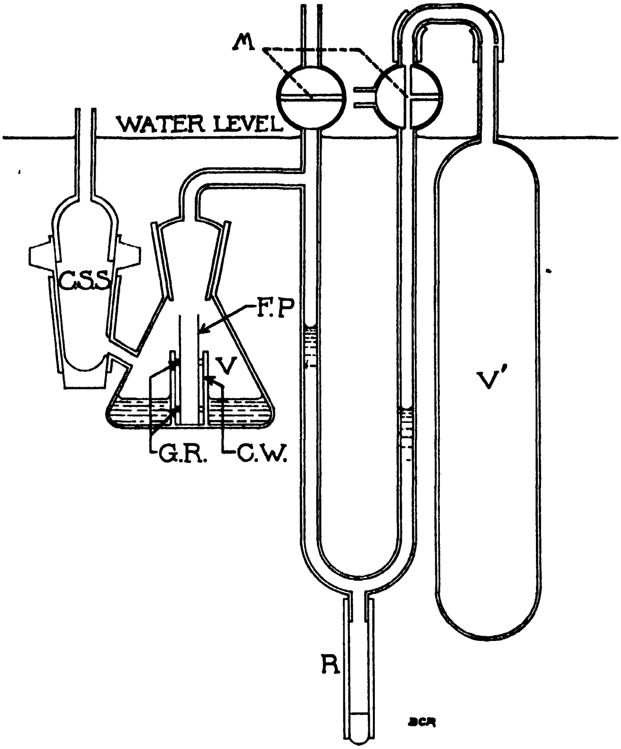

FIG. 5. Schematic drawing of the Warburg apparatus. The upper stop-cock *M*, shown in two sections, is actually built in one piece, with a long barrel. When mounted, the rubber tube *R* at the inferior end of the manometer is compressed at will by a simple screw-driven plate. A bar at the outer end of the screw engages the prongs of the control shaft, *13* in Fig. 1. *V* is the main vessel or respirator, and *V'* is the compensating vessel. (For a full discussion, see the text.) *C. S. S.* is the chambered side stop-cock; *F. P.* is a filter paper cylinder or roll; *G. R.* is two glass rings used to support the paper roll away from the sides of the center well, *C. W.*

Determination of Gas Exchange in Respirators

Fig. 5 shows diagrammatically the construction of the Warburg respirators, of which there are six units that can be simultaneously mounted in the pressure chamber.

Customarily when work is carried out at 1 atmosphere, the temperature being maintained nearly (±0.02°) constant, changes of barometric pressure and temperature are corrected for by the use of an empty vessel acting as a thermobarometer run in parallel with the experimental respirators. Such an arrangement at the high pressures of the chamber would be impracticable owing to the fact that relatively large changes in circumambient pressure are impossible to avoid, and also to the necessity of maintaining a slightly positive pressure in the chamber relative to that in the respirators, as outlined above. For this reason, the arm of the manometer not connected with the respirator is connected with a large *compensator* (V') of about 1000 cc. capacity by means of the main stop-cock (M, Fig. 5) which in the position shown connects the respirator and the compensator with the right and left arms of the manometer respectively, thus completely isolating them from the circumambient pressure. In practice the two actions are accomplished by a turn of 90° of a single stop-cock. In Fig. 5, the single stop-cock is divided for schematic purposes. The readings of the manometer are then unaffected by variations in the ambient pressure of the chamber as long as these are not great enough to cause leakage of the stopcock. Furthermore, since temperature is maintained constant at ±0.02°, the change in absolute amount of gas in each respirator can be measured in terms of the change in manometric heights without recourse to the thermobarometer ordinarily used.

The calculation of the change in gas volume in the respirator is derived as follows:

V = volume of the respirator to the 150 mark on the manometer corrected for the volume of medium, specimen, etc., placed within, cc.
V' = initial volume of the compensator to within 5 cc.
P_0 = " ambient pressure (respirator and compensator), mm. Brodie's solution
P, P' = any subsequent pressure, respirator and compensator respectively, mm. Brodie's solution
w = aqueous tension at 38°, mm. Brodie's solution
ΔG = change in the absolute amount of gas in the respirator at normal temperature and pressure, cc.
d, d' = change in level of the manometer on respirator side, or compensator side, respectively, mm. Brodie's solution

The sign of d and d' is determined by the convention, reading (initial) minus reading (final) = d (or d').

T = absolute temperature
10^4 = 1 atmosphere, mm. Brodie's solution
f = $273/T \times 10^4$, a factor correcting for temperature and converting mm. Brodie's solution to atmospheres

It is clear that after a finite period of respiration the *amount* of gas in the respirator changes, while that in the compensator remains unchanged. The *volume* of gas in the respirator changes by the amount *ad*, and that in the compensator by a corresponding amount *ad'* (with due respect to sign). The difference in hydrostatic pressure between respirator and compensator is obviously $(R^0_r - R^0_c) - (R_r - R_c) = (R^0_r - R_r) - (R^0_c - R_c) = d - d'$. R^0 and R are initial and final readings respectively of respirator (r) or compensator (c). From these considerations, the following primary equations arise.

$$(P - w) = (P' - w) + (d - d')$$

$$V'\ (P_0 - w) = (V' + ad')\ (P' - w);\ \text{hence}\ (P' - w) = \frac{V'}{V' + ad'}\ (P_0 - w)$$

$$\Delta G \times \frac{1}{f} = V\ (P_0 - v) - (V + ad)\ (P - w)$$

By combining and algebraic transformation,

$$\Delta G = fV\left\{(P_0 - w)\left[1 - \left(1 + \frac{ad}{V}\right)\left(\frac{V'}{V' + ad'}\right)\right] + \left(1 + \frac{ad}{V}\right)(d - d')\right\}$$

Since ad' is small relative to V', $V'/(V' + ad') = 1 - (ad'/V')$. Moreover, a/V is of the order of 10^{-4}. Hence, $a/V \times (d' - d)$ is negligible for values of d and d' in the range of the manometer. The above equation then simplifies to

$$\Delta G = F_2 d' + F_1 d \tag{3}$$

Where

$$F_1 = \left(1 + \frac{a}{V} P_0\right) k_{O_2} \tag{4}$$

$$F_2 = \left(1 + \frac{a}{V'} P_0\right) k_{O_2} \tag{5}$$

$k_{O_2} = (273/311)\ (1/10^4)V$ is the customary constant for the vessel. The solubility of oxygen in water is neglected. In measuring CO_2 the constant k_{CO_2} is substituted as in the manner customary for work at 1 atmosphere; *viz.*, $k_{CO_2} = k_{O_2} + A_{CO_2}S$ where α_{CO_2} is the solubility coefficient of CO_2 in cc. per cc. per mm. of Brodie's solution and S is the volume (cc.) of the aqueous phase in the respirator. ΔG may be converted from cc. to microliters or micromoles by multiplying by 1000 or 44.65 respectively.

In practice the readings of the manometers are arranged in horizontal columns as follows:

Time
Reading (compensator side) to nearest mm.
" (respirator ") " " 0.1 mm.
d'
$F_2 d'$
$F_1 d$
ΔG

The factors F_1 and F_2 are calculated by Equations 4 and 5 from the known values of a/V, a/V', k_{O_2}, and P_0. The values of a/V and a/V' are sufficiently close for a set of manometers, vessels, and compensators to permit use of average values. Frequently it is possible by adjustment of the manometer fluid to work at constant volume, *i.e.*, $d = 0$, thus simplifying calculations. It must be remembered, however, that whereas $1 + (a/V')P_0$ is of the order of 1.2 when the pressure is 8 atmospheres, the value of $1 + (a/V)P_0$ is 9. It is for this reason that d is read to 0.1 mm. and kept as small as possible without too much loss of time in the readings. In point of fact, however, it is easy to show with a constant amount of gas that wide variations in the level of the manometer fluid will give values of d and d' closely following Equation 3. Hence it is merely a matter of convenience whether to work at constant or variable volumes of gas in the respirator.

Experimental Procedure

The pressure chamber is filled with tap water and adjusted to 38°. The respirators containing the respiring system required by the nature of the experiment are equipped with filter roll and 0.2 to 0.25 cc. of N NaOH in the center well (see below). The chambered side cock (*C.S.S.*, Fig. 5) being empty, the vessels are attached to their respective manometers, and the vessels are all gassed at once with 100 per cent O_2 through a manifold for 3 to 4 minutes, the gas escaping through the open side cock. The side cocks are then closed and 1 cc. of sulfuric acid of appropriate concentration is introduced into each of them with a syringe and needle. This acid is subsequently, at the end of the respiratory period, admitted into the main vessel to terminate the respiration and to free any bound CO_2 in the tissues and medium for collection on the alkali filter roll. The respirator units are then fixed in position on their bearings (*8* and *9* in Fig. 1), the compensators are connected, the cover of the chamber is bolted into place, and the manometer fluid drawn down completely below the inferior turn of the manometers. This prevents the manometer fluid from being forced into vessel or compensator while the pressure is being rapidly raised. The pressure chamber is now flushed with a vigorous stream of oxygen for 5 to 7 minutes, sufficient to replace the initial air of the chamber by 98 to 100 per cent of oxygen (as determined by appropriate control tests).

Oxygen is now admitted through the inlet (*18*, Fig. 1) and the desired pressure having been reached, the manometer fluid is adjusted at some convenient level on the manometers, and the stop-cocks (*M*, Fig. 5) to the manometers and compensators are closed. Respirators and compensators are now isolated from the pressure chamber. The pressure gage (*A*, Fig.4) is now read and the stop-cock to the differential pressure gage (*B*, Fig. 4) is closed. The pressure in the pressure chamber is now raised by about 50 mm. of Brodie's solution as determined by the reading of the differential pressure gage. 10 minutes are allowed to elapse for the attainment of the physical equilibirum between tissue and aqueous phase, with the high pressure gas phase. Readings are then begun and repeated as occasion requires. At the end of the desired respiratory period, the acid in the chambered side stop-cocks is admitted into the respirators, as mentioned above, by means of control *11* (Fig. 1) to terminate the reaction, and 20 minutes are allowed for the absorption of all CO_2 upon the filter roll. All stop-cocks are then opened and the pressure is brought down to 1 atmosphere. The cover is removed, the respirators are disconnected, and the alkali filter rolls quantitatively removed, and the CO_2 output determined as described below.

Range of Method

The method permits at pressures of oxygen up to 8 atmospheres (the maximum safe pressure for which this apparatus was built) the determination of the oxygen uptake of tissue slices, homogenates, etc., with the simultaneous determination of the carbon dioxide output. With appropriate controls to determine the CO_2 of parallel systems at the beginning of the respiratory period, the respiratory quotient can be calculated. Various enzyme systems can also be studied under high pressure and, if desired, the chambered side stop-cock may be used for the introduction at high pressures of substrate, auxiliary substances, etc.

Determination of CO_2 Output

The determination of the CO_2 output of tissues when the manometers are enclosed in a high pressure chamber requires a different technique from that customarily used when one is working in the open at 1 atmosphere. In the latter case the CO_2 absorbed by the alkali absorbent during the respiratory period is determined in the same repirator following acidification of the alkali. Alkali and acid being initially in separate compartments, mixing is easily accomplished by tipping the respirator unit after removal from the bath. This is obviously impossible with the high pressure apparatus. For this reason the acid is introduced into the medium from the chambered side stop-cock (*C. S. S.*, Fig. 5) by manipulation of control *11* (Fig. 1). This serves to kill the tissue and release any CO_2 present in the medium as bicarbonate. 20 minutes are allowed to elapse for the absorption of this CO_2 and the CO_2 present in the gas phase during the respiratory period. The apparatus being then disassembled, the filter roll is removed, with quantitative precautions, and placed in a clean Warburg respirator of the ordinary type. The center well of the experimental respirator is carefully washed four times with 0.5 cc. portions of water, the washings being added to the vessel containing the filter roll. This vessel is then equipped with the requisite quantity of acid in its side sac and set up in a bath. After temperature equilibration, acid and alkali are admixed and the CO_2 determined by the customary technique. As is customary when the net CO_2 formed during a given respiratory period is to be determined, a system similar to the experimental one is set up in parallel. This is acidified at zero time of the respiratory period and its CO_2 content determined as above. This "initial CO_2" is applied as a correction to the total CO_2 of the respiring sample to give the net CO_2 formed during the period. The respiratory quotient can then be calculated.

Rate of Absorption of CO_2 at High and Low Pressures

It was to be expected on general theoretical grounds that the rate of diffusion, and hence the rate of absorption, of CO_2 from the medium to the alkali filter roll should be markedly influenced when the total pressure is increased by addition of an indifferent gas. This was found actually to be the case and necessitated care in the arrangement of the filter roll to obtain adequate rates of absorption. To compare rates of absorption under given physical conditions, use was made of the fact that absorption follows a first order course, *viz.* 2.3 log $P_{CO_2} = kt +$ constant, where P_{CO_2} is the partial pressure of CO_2 at any given time, t, during the process of absorption. k is a measure of the rate of absorption and is the proportion of the CO_2 present at any time absorbed in a unit of time. With any given physical set-up, CO_2 was introduced into the vessel usually by the acidification of a contained bicarbonate solution. The initial pressure of CO_2 was on the average about 150 mm. of Brodie's solution. After a minute or two to allow complete evolution of the CO_2, manometer and time readings were made until no more absorption occurred. The P_{CO_2} values thus obtained followed the above equation closely in most instances. From the slope of the log

TABLE I

Rates of Absorption of Carbon Dioxide in Warburg Respirator under Varying Conditions (Temperature, 38°)

Total pressure	Arrangement for CO_2 absorption	k, reciprocal log min.
atmospheres		
1	Filter roll, 30 × 22 mm., in center well extending 1 mm. below top of center well; 0.2 cc. N alkali	0.16
8	Same	0.048
1	Filter roll, 30 × 37 mm., in center well extending 15 mm. above top of well	0.61
8	Same	0.21

P_{CO_2} plot against time, *k* could be calculated. Many determinations were made with consistent results. A few are given for discussion.

It will be seen from Table I that with the same physical set-up and the same degree of shaking, the rate of absorption of CO_2 is reduced by one-third when the total pressure is increased by a diluting gas from 1 to 8 atmospheres.

The conditions finally selected were more than adequate for CO_2 absorption at oxygen pressures up to 8 atmospheres. A piece of filter paper (30 × 37 mm.) was held in the form of a cylinder containing one thickness of paper only by means of two small glass rings. This assembly is inserted into the center well (*G. R.* and *F. P.*, Fig. 5) The glass rings prevented contact of the filter roll with the walls of the center well. This arrangement increased the effective surface and also prevented alkali from creeping into the main vessel. Rubber rings cannot be used, since rubber absorbs oxygen when the pressure is greatly increased. Just enough alkali (0.2 to 0.3 ml.) of appropriate strength (usually 4 to 5 times the equivalent of expected CO_2 evolution) is added to the center well to moisten the filter roll.

Additional Factors in Gasometric Analyses

Since a large part of the effect of high oxygen pressure upon the metabolic systems might reasonably be expected to occur early in the period of exposure, it became necessary to determine the minimum length of time required for the system to come to physical equilibrium when the pressure of oxygen is suddenly increased from 1 to 8 atmospheres, the highest pressure used. With the present apparatus this pressure can be attained in about 2 minutes. A number of experiments were therefore performed in which the pressure was rapidly raised to 8 atmospheres. Since the vessels contained no tissue, the time required for the attainment of physical equilibrium could be measured. In some cases the vessels contained the appropriate medium for the study of tissues, and in others slightly acidified water was used in order to eliminate the absorption of the small amount of CO_2 inevitably present. Constant readings were always obtained after a maximum of 8 minutes from the time maximum pressure was reached, and in many cases in less than 2 minutes. In practice, therefore, readings are begun 8 minutes after the pressure has attained its maximum. The attainment of temperature equilibrium presented no problem, because of the additional time spent in flushing the apparatus with oxygen before the pressure was increased (5 to 7 minutes), during which the vessels were being constantly shaken in the water bath at 38°.

A further difficulty presented itself at high pressures of oxygen. Rubber dissolves appreciable amounts of oxygen. Hence when suddenly brought to a new high pressure, oxygen is absorbed by the rubber and the attainment of equilibrium is a slow one (30 to 60 minutes). It was therefore necessary to connect the static compensators to the shaking manometers with carefully fitted glass connections, united by rubber tubing in such a way that a negligible amount of rubber surface was presented to the gas phase inside the manometer units. A preliminary attempt to use rubber tubing alone resulted in a slow change in gas volume on the compensator side sufficient to cause a serious error in experimental measurements, with equilibrium not reached at the end of an hour.

Another consequence of having the compensating vessels not shaken (as dictated by their size and the space available, as well as mechanical complications) is the relatively long time required for physical equilibrium of any water contained in them beyond barely visible moisture. Precautions are therefore necessary to prevent water from accidentally accumulating. This is particularly important when respiration is measured at a low pressure following exposure to high pressure; in this case, equilibrium is even more slowly attained.

SUMMARY

1. An apparatus and technique are described for the measurement of gaseous metabolism *in vitro* of surviving tissues, enzymatic systems, etc., under pressures of oxygen up to 8 atmospheres, absolute.

2. The apparatus consists of a cylindrical high pressure chamber which contains six Warburg respirators and manometers. The scales of the manometers are read through windows and the necessary manipulations are performed by means of external controls.

3. The main structural features of the apparatus are given in full.

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MICROTOME FOR THE PREPARATION OF TISSUE SLICES FOR METABOLIC STUDIES OF SURVIVING TISSUES IN VITRO*

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(Received for publication, May 10, 1944)

A major requirement for metabolic studies of tissues *in vitro* with the Warburg or similar apparatus is the preparation from sundry organs of thin slices of uniform thickness. When economy of material is necessary as in the case of the small organs of the rat, this single operation often becomes a limiting factor in the amount of material available. Several methods are currently used in this operation, of which the commonest are the free-hand method with a razor, with the subsequent selection of suitable portions of the slices, and various forms of double bladed cutters.

Both of these methods are relatively wasteful of material, particularly with small organs such as rat kidney or brain. Furthermore, in precise metabolic studies it is important to know that the thickness of the given slices is below the "limiting thickness" necessary for maximum oxygen uptake and metabolic activity. Above this point, the gas exchange is limited to an increasing extent by the thickness of the slice, and becomes less and less a measure of true metabolism. For this reason, the thickness of free-hand slices often must be individually determined by measuring their areas and weights. At the same time, it often occurs that the limiting thickness of a tissue is such that a slice appreciably thinner becomes difficult to prepare by these methods. This is particularly true of friable tissue such as brain. For metabolic studies the use of fixative or freezing agents is, of course, prohibited.

A microtome for such slice preparations from fresh tissue is here described. In principle, the device mechanically holds a definite thickness of tissue between the under surface of a transparent plate and the advancing edge of a thin, flat razor blade. In this way multiple slices can be quickly made with a minimum of trauma and with considerable reproducibility. The accompanying working drawings[1] should be largely self-explanatory, but the specifications are summarized for convenience.

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania. Permission for publication granted by the Office of Scientific Research and Development.

[1] Enlarged copies of all figures may be obtained upon request from the authors.

Fig. 1 represents the microtome, which is constructed of two pieces of 5/16 inch Plexiglas, fastened with brass or stainless steel screws carefully centered for interchangeable, end for end assembly. These are tightened only to bare holding tension (in practice, with a finger nail) to minimize cold flow of the plastic.

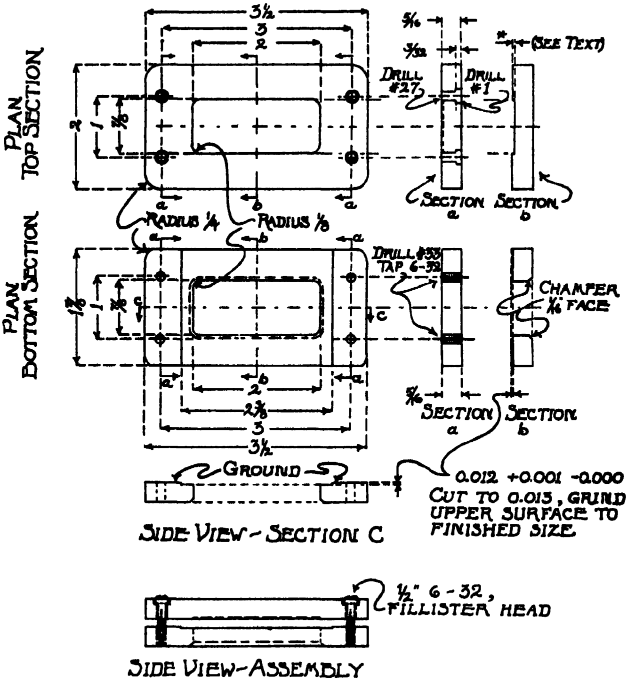

Fig. 1. Working drawing of the microtome. All dimensions are in inches

Table I

Working Dimensions of Microtomes for Various Thicknesses

Tissue thickness required		Depth of cut	Finished depth (tissue thickness less one-half blade thickness)
mm.	*inch*	*inch*	*inch*
0.25	0.0098	0.008	0.0048
0.35	0.0138	0.012	0.0088
0.50	0.0197	0.018	0.0147
0.75	0.0295	0.028	0.0245

The upper surface of the top section is ground and polished flat with full transparency (except for minor scratches). The 2 × ⅞ inch depression in the lower surface is machine-cut to a depth greater than required and brought down to the exact depth (depending on the slice thickness desired) by hand grinding the uncut surface on a steel table to ±0.0005 inch. Table I shows these dimensions for various thicknesses of slice. The instrument is designed for use with 4 inch lengths of unperforated Old Fashioned Valet Type Autostrop Blade,[2] whose thickness is 0.010 inch within extremely close limits.

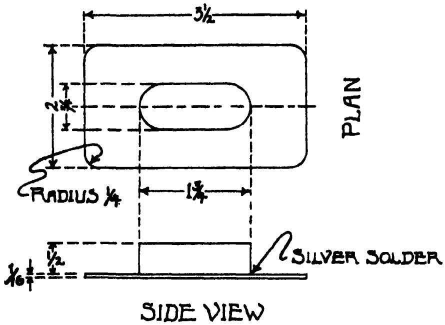

Fig. 2

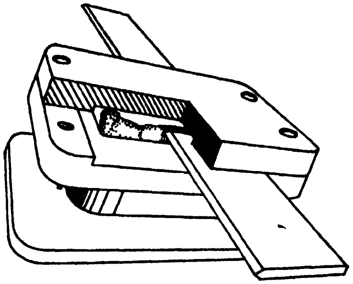

Fig. 3

Fig. 2. Working drawing of base for holding tissue. The dimensions are in inches.

Fig. 3. Diagrammatic perspective view of completed microtome, with tissue and blade in place, partly cut away. The thicknesses of the blade and slice are exaggerated for clarity.

Table II

Average Thickness of Slices from Microtome Built to Cut 0.50 Mm.

Organ	No of slices	Mean thickness	Standard error
		mm.	*mm.*
Liver	6	0.51	±0.022
Kidney	8	0.47	±0.024
Heart	6	0.49	±0.024
Brain	6	0.49*	±0.019

* Calculated from dry weights (see the text).

The upper surface of the bottom section has a similar depression machine-cut and ground to the dimension shown in the drawing, which affords passage to the blade with 0.001 inch clearance. The center is cut out to allow entrance of the tissue.

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[2] These blades were secured from the Gillette Safety Razor Company, Boston, Massachusetts, in uncut lengths of 4 inches, through the courtesy of Mr. Stafford Johnson, to whom we are indebted.

Fig. 2 shows the base, which is merely a small table built of stainless steel to hold the tissue.

In operation, the tissue rests on a moistened bit of filter paper on the steel table. The blade is introduced into the microtome, which is then gently pressed down on the surface of the tissue. The blade is advanced with a to and fro motion until the slice is complete, and is then removed. The slice may be washed up from the inverted microtome in a dish of saline, or lifted out with a spatula. The operation can be repeated with ease and rapidity. Fig. 3 shows the instrument in perspective, partly cut away, with tissue being sliced. (The thickness of blade and slice are exaggerated for clarity.)

Table II shows the average thickness of slices from several rat organs made with the cutter built theoretically to cut 0.50 mm. thickness. The standard error in all cases can be seen to be within 5 per cent. The method of measurement was as follows: The fresh slice was floated over mm.-squared paper, and the area taken by counting the uncovered squares within a given area, fractions being estimated to 0.5 sq. mm. In some cases the areas were taken by trimming a piece of filter paper accurately to the size of the slice and subsequently taking its area with a planimeter or by the use of squared paper. The slices were then blotted dry and weighed on a torsion balance to the nearest mg. The density of all tissues was assumed to be 1.04.

Preparation of slices from the brain, particularly from the rat, is usually difficult. The extreme friability of the tissues makes blotting, for determination of the moist weight, or excessive handling inadvisable. With the microtome we have found the preparation of uniform slices relatively easy (see Table II). In practice, we have floated the slices out of the inverted microtome with saline and transferred them to the respiratory vessels with a spatula. The dry weights were determined at the end of the respiratory period. Dry weight to wet weight ratios were obtained by blotting a large piece of the discarded portion of the brain, weighing, and drying.

Slices of other organs, including liver, kidney, lung, and heart, are prepared routinely with great ease and rapidity. For example, from a single rat kidney, four to six pieces 0.5 mm. thick and weighing 50 to 75 mg. each may be easily cut.

SUMMARY

1. A microtome for the preparation of thin slices of fresh tissue for studies of metabolic activity *in vitro* is described. Working drawings and specifications are included.

2. Representative data as to the accuracy and reproducibility of slices prepared by the apparatus are given.

STUDIES ON THE COMPOSITION OF RICKETTSIA PROWAZEKI*

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(Received for publication, May 16, 1944)

The study presented here was undertaken at the request of Colonel H. Plotz following observations on the distribution of rickettsial antigens made in the laboratory of the Division of Virus and Rickettsial Diseases, Army Medical School, Washington, D. C. The primary objective was the collection of chemical information on the presence and properties of specific substances in different fractions of the rickettsial cell, disintegrated by a variety of methods, and in certain by-product fluids obtained during the purification of the organisms cultivated in the yolk sac of the developing chick embryo, according to the method of Cox (1).

In contrast to the large amount of information on the chemical composition of pathogenic bacteria, our knowledge of the chemistry of rickettsiae is extremely scanty. This has been primarily due to the difficulty of cultivating these obligate intracellular parasites in amounts sufficient for chemical work. Some data on rickettsial antigens, with special reference to their relation to the Weil-Felix reaction (2, 3), and on rickettsial toxins (4, 5) will be found in the literature. With regard to the classification of rickettsiae and to the epidemiological and immunological aspects of epidemic typhus and related diseases reference may be made to a number of recent summaries (6–10).

The organism used in the present study was *Rickettsia prowazeki*, the etiological agent of epidemic typhus. The material, grown by the method of Cox (1), had been treated with formaldehyde previous to its fractionation; we are highly indebted for it to Colonel H. Plotz and his associates at the Virus Laboratory of the Army Medical School.

The investigation of the specific components of purified rickettsiae is made difficult by the scarcity of the available material. It furthermore suffers from the common limitations of work on bacterial constituents; *viz.*, the lack of well understood and generally applicable procedures. The uneconomical method of trial and error has to be followed throughout. It is perhaps worthy of note that many methods used successfully for the isolation of specific bacterial fractions, *e.g.* the treatment of aqueous suspensions

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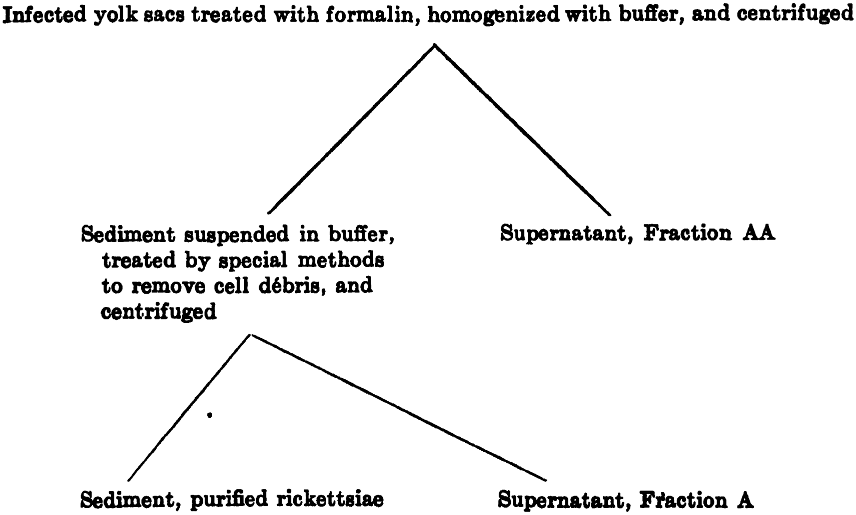
* This work was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

of bacteria with chloroform, trichloroacetic acid, or proteolytic enzymes, are based on the alteration, degradation, or destruction of the bacterial proteins. One feature of the present work to which attention may be drawn is the extensive use of physical methods for the separation of antigenic fractions.

EXPERIMENTAL

Preparation of Rickettsiae

The accompanying flow sheet indicates the methods adopted at the Division of Virus and Rickettsial Diseases, Army Medical School, for the preparation of the material supplied to this laboratory.



Infected yolk sacs treated with formalin, homogenized with buffer, and centrifuged

Sediment suspended in buffer, treated by special methods to remove cell débris, and centrifuged

Supernatant, Fraction AA

Sediment, purified rickettsiae

Supernatant, Fraction A

In the present study several preparations of purified epidemic rickettsiae (representing a total of 1.9 gm.) and numerous preparations of the by-product fluids, *viz.* Fractions AA and A, were examined.

Estimation of Specific Activity

The purification of rickettsial antigens depended on the availability of a rapid method for the demonstration of specific activity. Rabbit antisera against formolized epidemic rickettsiae contain rickettsial agglutinins in high titer, but with isolated rickettsial fractions the conditions for obtaining specific precipitation appeared less favorable because of the apparently low combining ratio of antibody to antigen. With the rickettsial antisera available, satisfactory precipitation of individual rickettsial fractions could be observed only at a comparatively high concentration of both antigen and antiserum, although the same sera exhibited high titers in the agglutination of intact rickettsiae. The dilution of the antigens and the antisera resulted in the appearance of non-flocculent turbidities. Since, moreover, the solutions of several antigens examined were opalescent, the application of this precipitin reaction was far from inviting; but it proved useful in the course of many experiments and its results agreed well with those of complement fixation tests later performed with the same fractions at the Army Medical School.

The tests were carried out by mixing 0.1 cc. of the rabbit antiserum (diluted with 2 parts of physiological saline) with 0.1 cc. of the antigen dilution in saline. All solutions contained 0.01 per cent of ethyl mercurithiosalicylate. The customary saline and serum controls were included. The mixtures were stored at 37° for 2 hours and at 4° for 24 hours. The degree of turbidity was then estimated before a strong lamp and the end-points were checked by examining the sediments after the tubes were centrifuged for 5 minutes at 2500 R.P.M. When new antisera were to be employed, it was first ascertained that the end-points of previously examined antigen solutions remained unaffected. Guinea pig antisera were found unsuitable for these tests.

Fraction AA

Three preparations derived from separate harvests of infected yolk sacs and a fraction from normal non-infected yolk sacs (prepared under strictly comparable conditions) were examined. The highly opalescent lipid-containing emulsions were partly clarified by extraction with ether and tested for typhus-specific precipitating activity. No activity was observed in the unconcentrated preparations or in supernatants clarified in the ultracentrifuge. One fraction which had been concentrated by pervaporation to one-seventh of its original volume showed a slight precipitating activity when tested with a rabbit antiserum against epidemic rickettsiae. The amounts of specific material present in Fraction AA appear to be very slight, probably not more than one-tenth of those found in fresh preparations of Fraction A, to be discussed later, which generally were found reactive before concentration.

For a comparison between Fractions AA from normal and infected yolk sacs the solutions were extracted with ether and the aqueous layer subjected to a centrifugation at 7500 R.P.M. for 30 minutes. The electrophoretic examination of the supernatants in the Tiselius apparatus at pH 7.1 revealed the presence of two main components in both the normal and the infected preparations. The former appeared considerably richer in proteins (compare Table I). The slow electrophoretic component had a sharp boundary, was highly opalescent, and readily sedimentable, when subjected to centrifugation at 42,000 R.P.M. for 40 minutes. This fraction was almost completely precipitated at pH 4 to 5. The faster component, likewise readily sedimentable in the ultracentrifuge, was non-opalescent. When stored at 4° for several weeks, it decomposed to form products which were no longer readily sedimentable in the ultracentrifuge. The faster component was soluble over a wide pH range. The mobilities and relative proportions of these components are indicated in Table I.

The injection into rabbits of a rickettsial preparation containing Fraction AA resulted in the production of antisera containing a considerable amount of precipitins for normal egg antigens. Following the absorption of the non-specific antibodies with normal egg yolk sac material, these antisera may be used for the estimation of rickettsia-specific activity. The bulk of antibodies apparently was not rickettsia-specific, but reacted with the opalescent slow component from normal yolk sac preparations.

TABLE I

Electrophoretic Properties of Fraction AA

The experiments were carried out in 0.02 M barbiturate buffer containing 0.08 M NaCl.

Source	N per 1 cc.	pH	Descending boundaries		Ascending boundaries	
			Mobilities	Area	Mobilities	Area
	mg		$u \times 10^5$	*per cent*	$u \times 10^5$	*per cent*
Normal yolk sacs	4.4	7.1	−3.7	45	−5.0	40
			−5.5	55	−6.8	60
Yolk sacs inoculated with *Rickettsia prowazeki*	0.45	7.2	−4.8	30	−5.3	30
			−6.6	70	−6.4	70

Fraction A

Isolation of Specific Material by Fractional Precipitation and Sedimentation—The following properties of the soluble specific substance (Fraction AB) could be used for its isolation from the starting material which was obtained as a colorless opalescent solution.[1] It is soluble at pH 4 to 5 at a low electrolyte concentration; it is insoluble at 50 per cent saturation with ammonium sulfate at 4°; it can be sedimented by centrifugation at 48,000 R.P.M. for 90 minutes; it appears as a relatively slow moving component, when subjected to electrophoresis between pH 7 and 8. More than half of the active material present in Fraction A could be isolated by a combination of these methods.

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[1] It is advisable to isolate the soluble antigenic fraction as soon after harvesting as possible, since it appears to be destroyed on prolonged storage. All operations described here were, as far as possible, carried out in the cold.

The A fractions were dialyzed and concentrated by pervaporation to between 7 and 10 per cent of the original volume. The precipitate which separated, when the concentrated solution was adjusted to 50 per cent saturation with ammonium sulfate, was dissolved in water and the solution, whose volume was one-half that of the concentrate, brought to pH 5. The precipitate appearing at this point had only slight activity and was discarded after centrifugation. The specific soluble substance was again precipitated from the supernatant by half saturation with ammonium sulfate and dissolved in physiological saline (one-seventh to one-tenth of the volume of the original concentrate). After dialysis, the solution was stored for 2 weeks at 4°, in order to decompose the normal heavy egg yolk component mentioned in the preceding section, and then subjected to centrifugation at 48,000 R.P.M. for 90 minutes. The brown translucent pellets slowly dispersed in saline to yield slightly opalescent colorless solutions from which, after preliminary centrifugation at 4000 R.P.M. for 30 minutes, they were again sedimented in the ultracentrifuge.

The preparations thus isolated, designated Fraction AB, gave specific precipitation with rabbit antisera against *Rickettsia prowazeki* and *Rickettsia mooseri* at end-points of 1.0 to 1.5 γ and 4.0 to 6.0 γ of antigen N per cc. respectively. They did not react with rabbit antisera against egg albumin or normal yolk sacs. About 50 to 75 per cent of the specific precipitating activity present in the starting material could be isolated in this manner. The yield of the purified Fraction AB in terms of rickettsial nitrogen may be exemplified by the following typical balance. From 450 inoculated yolk sacs purified rickettsiae containing a total of 48.6 mg. of rickettsial N were harvested. In the same experiment 3800 cc. of Fraction A were obtained, which yielded 1.8 mg. of N (3.7 per cent of the rickettsial N) in the form of the soluble antigen.[2]

A purified specimen of Fraction AB, examined in the Tiselius electrophoresis cell at pH 6.8 in 0.02 M barbiturate buffer containing 0.08 M NaCl, exhibited a single boundary with a mobility of -5.3×10^{-5} sq. cm. per volt per second on the ascending side and of -4.4 on the descending side. The substance proved heterodisperse in the analytical ultracentrifuge. The extremely small amounts available precluded a detailed chemical characterization of this material, but similar fractions obtained from Fraction A by electrophoretic separation will be discussed later in this paper.

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[2] According to private communications from the Division of Virus and Rickettsial Diseases of the Army Medical School, 8 and 15 γ of N per cc. of this fraction fixed 4 units of complement with human epidemic and endemic convalescent sera (11) respectively. Twelve guinea pigs, each receiving a total of 32 γ of antigen N in two injections, produced specific complement-fixing antisera (pooled) of low titer (1:5) for epidemic rickettsiae. Seven of ten of these guinea pigs were partially or completely protected against a challenge dose which produced typhus in all control animals. The intravenous injection of 0.27 mg. of total antigen N into several rabbits produced precipitins for the antigen, a high agglutinin titer (1:640) for epidemic rickettsiae, and a low agglutinin titer (1:20) for endemic rickettsiae. No precipitins for normal yolk sac suspensions and egg albumin were produced.

TABLE II

Electrophoretic Separation of Fraction A

Experiment No.	Fraction	N	P	N:P, atomic ratio	Ultraviolet absorption maximum	Pptn. end-point		Electrophoretic mobilities	
						Rabbit antiserum against epidemic rickettsiae	Rabbit antiserum against endemic rickettsiae	Descending boundaries	Ascending boundaries
		per cent of original	*per cent of original*		*A*	*γ N per cc.*	*γ N per cc.*	$u \times 10^5$	$u \times 10^5$
1	A, concentrate (barbiturate buffer, pH 7.0)	100	100	12	2660	6.5	13	−4.1, −5.0, −6.7, −14.0	−3.4, −4.4, −6.5, −13.5
2	A, concentrate (borate buffer, pH 7.8)							−4.0, −5.9, −7.6, −15.6	−4.3, −6.1, −8.4, −15.8
3	AF	48	88	5	2600	18	>18	−14.0	−13.5
4	AS	52	12	44	2750	2.5–5	10	−4.1, −5.0, −6.7	−3.4, −4.4, −6.5
5	AS-P	36	10	30		7.5	7.5		
6	AS-S (borate buffer, pH 7.8)	16	2	66		1	4	−5.6	−5.1

The incubation of solutions of the antigen at 37° for 24 hours with crystalline trypsin or chymotrypsin[3] at pH 7.8 did not affect the end-point of antigen dilution at which specific precipitation was observed. The solutions were also stable to heating at 100° for 1 hour, and retained their precipitating properties, when kept over a pH range of 3 to 10 for 24 hours at 4°.

Electrophoretic Fractionation—One preparation of Fraction A was concentrated by pervaporation to about 3 per cent of the original volume and dialyzed against water. Portions of the concentrate, containing 520 γ of N and 95 γ of P per cc., were subjected to electrophoretic fractionation in the Tiselius cell in 0.02 M barbiturate buffer (containing 0.08 M NaCl) at pH 7.9 and in 0.1 M borate buffer of the same pH. The material was separated into one fast component (Fraction AF) and a group of three slower components (Fraction AS). Data on these experiments will be found in Table II, where the separation experiments are presented as Experiments 1 and 2, while the properties of the separated fractions are summarized as Experiments 3 and 4. The ultraviolet absorption spectra of the original concentrate of Fraction A and of dialyzed Fractions AF and AS, obtained by means of a Hilger spectrograph, are reproduced in Fig. 1.

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[3] We are indebted to Dr. M. Kunitz of the Rockefeller Institute, Princeton, New Jersey, for these enzyme preparations.

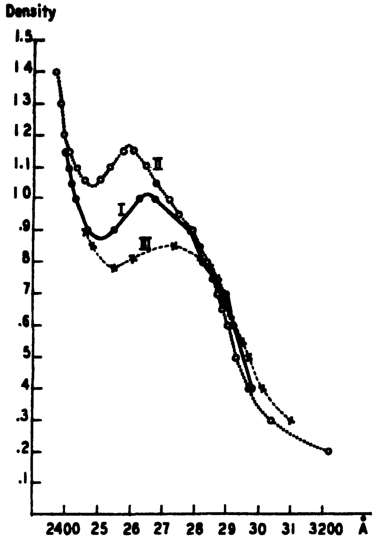

FIG. 1. Ultraviolet absorption spectra of Fraction A (concentrated) (77 γ of N per cc.), Curve I; Fraction AF (60 γ of N per cc.), Curve II; Fraction AS (66 γ of N per cc.), Curve III.

Approximately 90 per cent of the total phosphorus contained in the concentrate of Fraction A resided in the fast component, Fraction AF, which appeared to consist principally of nucleic acid. (Compare Fig. 1 and Table II, Experiment 3.) The ultraviolet absorption spectrum showed a maximum at 2600 A. The atomic N:P ratio of 5 (required by the theory, 3.75) indicates the presence of some contaminating nitrogenous material.[4]

[4] The electrophoretic separation of a concentrated preparation of Fraction A from a harvest of endemic rickettsiae (*Rickettsia mooseri*) similarly revealed the presence of considerable amounts of free nucleic acid, whereas in a comparable preparation from normal yolk sacs no free nucleic acid could be demonstrated.

The group of slow electrophoretic components, Fraction AS (Table II, Experiment 4), which retained the specific activity of the starting material was subjected to further purification. When the solution of Fraction AS was brought to pH 4 to 5 by the addition of dilute acetic acid, precipitation of Fraction AS-P took place (Table II, Experiment 5). The nonopalescent supernatant contained Fraction AS-S (Experiment 6) which showed considerable precipitating activity. This material appeared similar, both in electrophoretic mobility and in specific activity, to the previously discussed soluble specific substance (Fraction AB) isolated from Fraction A by salt precipitation and high speed sedimentation.

Rickettsial Organisms

Electrophoretic Mobility of Intact Rickettsiae—In order to test the homogeneity of formolized rickettsiae, four separately harvested preparations were examined over a wide pH range in uniunivalent buffers (ionic strength 0.02) in the micro electrophoresis apparatus described by Northrop and Kunitz (12).[5] All specimens examined behaved almost identically, although prepared in two different laboratories, and between 90 and 95 per cent of the particles of the individual preparations exhibited uniform mobilities. The average mobilities (in μ per second per volt per cm.) were +1.35 at pH 1.75, +1.01 at pH 2.57, 0 at pH 3.40, −2.38 at pH 4.51, −3.08 at pH 6.17, and −3.89 at pH 7.92. The shape of the mobility curve and the spread of the individual values are indicated in Fig. 2.

Lipids—A specimen of purified rickettsial organisms was washed with distilled water and dried in a high vacuum in the frozen state. The organisms contained N 12.2 per cent (Dumas) and P 0.93 per cent (Pregl-Lieb). For the removal of lipids 120.6 mg. of the dry organisms were extracted for 14 hours with 50 cc. of a boiling mixture of equal parts of absolute alcohol and ether. The extraction residue was filtered off and washed with absolute alcohol. The combined filtrates, after evaporation *in vacuo*, yielded the *total lipids*, weighing 16.0 mg. (13.3 per cent of the rickettsiae). The acetone-insoluble phospholipid fraction was obtained by the addition of acetone to a solution of the lipids in alcohol-ether; it weighed 3.7 mg. (3.1 per cent of the rickettsiae). The acetone-soluble lipid fraction was recovered from the mother liquor; it weighed 11.5 mg. (9.5 per cent).[6]

Extraction of Specific Substances—The treatment of the rickettsiae with physiological saline, 0.005 N NaOH, N acetic acid, or 2.5 M urea resulted in the extraction of only very small amounts of rickettsia-specific material.

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[5] We should like to thank Dr. M. Kunitz of the Rockefeller Institute, Princeton, New Jersey, for permission to use the micro electrophoresis apparatus.

[6] According to a private communication from Colonel H. Plotz, Army Medical School, these lipid fractions failed to fix complement with epidemic typhus convalescent sera.

Dry rickettsiae were shaken with a 50-fold weight of diethylene glycol (in the presence of glass beads) for 24 hours at room temperature. The extraction residues, collected by centrifugation, had lost between 20 and 30 per cent by weight and about 10 per cent of the total nitrogen. The supernatant was freed of the solvent by prolonged dialysis through cellophane against running and distilled water and the resulting aqueous solution freed of lipids by extraction with ether. Under these circumstances, the aqueous phase was found to contain 1 to 2 per cent of the total rickettsial nitrogen. The extracted material showed a precipitation end-point at 0.7 to 0.9 γ of N per cc., when tested with rabbit antisera against epidemic or endemic rickettsiae or against the specific soluble material isolated from Fraction A.

Isolation of Specific Fractions from Disintegrated Rickettsiae—The disintegration of the rickettsial organisms with proteolytic enzymes was found to produce a larger yield of antigenic material. The rates of rickettsial breakdown, followed by means of electrophotometric turbidity measurements, and the yields and properties of the liberated fractions were similar, when commercial trypsin (Fairchild), crystalline trypsin,[3] or crystalline chymotrypsin[3] in 1/15 M phosphate buffer of pH 7.8 was employed. Lysozyme preparations (from egg white) had no visible effect. Some of the digestion experiments were carried out with residues from the diethylene glycol extraction.

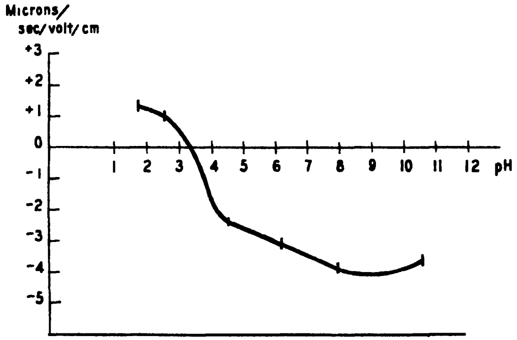

FIG. 2. Electrophoretic mobility of preparations of *Rickettsia prowazeki*. The spread of the individual values observed with four different preparations is indicated by the vertical lines.

In a typical experiment, 70 cc. of an aqueous suspension of the rickettsial bodies (0.7 mg. of N per cc.) were adjusted to pH 8.3 and incubated with 7 mg. of trypsin (Fairchild) and 3.5 cc. of toluene at 37° for 3 days. Most of the suspended material disappeared during this period, to form an opalescent viscous solution. A slight insoluble residue was removed by centrifugation and the supernatant subjected to dialysis, when 65 per cent of the original nitrogen was obtained in non-dialyzable form. (The digestion with crystalline trypsin yielded slightly more non-dialyzable N.) The precipitation end-point of this material with rabbit antisera against epidemic or endemic rickettsiae was found to be 1.4 γ of N per cc.

The solution of the digested material was subjected to electrophoretic separation into two distinctly migrating components (see Table III). Following separation, the individual fractions proved almost homogeneous electrophoretically; it required a 10-fold concentration to demonstrate the presence of very small amounts of the contaminating component. The fast electrophoretic component, Fraction RF, represented (in terms of nitrogen recovery) about 70 to 80 per cent of the material obtained by enzymatic digestion; *i.e.*, about 45 to 50 per cent of the rickettsial organisms. The slow component, Fraction RS, therefore, corresponded to about 15 per cent of the rickettsiae.

TABLE III

Rickettsial Components Following Proteolytic Digestion

Experiment No.	Enzyme	Electrophoretic mobilities*						Pptn. end-points†	
		Fraction RF		Nucleic acid		Fraction RS		Fraction RF	Fraction RS
		Descending	Ascending	Descending	Ascending	Descending	Ascending		
								γ N per cc.	*γ N per cc.*
1	Trypsin (Fairchild)	−16.8	−18.0			−6.1	−6.8	1.5	1.4
2	Chymotrypsin (crystalline)	−17.9	−18.3			−4.5	−4.9	1.3	1.1
3	Trypsin (crystalline)	−17.6	−16.7	−14.9	−14.2	−5.8	−5.9	0.8–1.6	1.0–2.0

* The experiments were carried out in 0.1 M phosphate buffer of pH 7.0. The mobilities are expressed as 10^{-5} sq. cm. per volt per second.

† Tested with rabbit antisera against rickettsiae.

Electrophoretic and serological data, obtained in several experiments, are summarized in Table III.[7]

Properties of Fractions Separated by Electrophoresis—Both electrophoretic components, *viz.* Fractions RF and RS, were readily sedimented in the ultracentrifuge, but appeared to be inhomogeneous with respect to particle size. For the isolation of these fractions, the electrophoretic separation was followed by thorough dialysis of the solution and evaporation of the water in the frozen state in a high vacuum. The samples for analysis were dried *in vacuo* over P_2O_5 at 61°. Analytical data on the contents of nitrogen, carbohydrates (by the orcinol method) (13, 14), total reducing sugars (15), and amino sugars[8] (16) will be found in Table IV. For the determination of reducing sugars, the samples were hydrolyzed in sealed tubes with N HCl at 100° for 4 hours, which proved the optimum time. In both fractions the

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[7] According to private communications from the Division of Virus and Rickettsial Diseases of the Army Medical School, 1.25 and 2.5 γ of N per cc. of both Fractions RF and RS fixed complement with human epidemic and endemic convalescent sera respectively. Twelve guinea pigs, each receiving a total of 20 γ of antigen N of either Fraction RF or RS in two injections, produced complement-fixing antisera (pooled) of low titer (1:12), specific for epidemic rickettsiae. 70 per cent of these animals, immunized with either Fraction RF or RS, was protected against epidemic typhus.

TABLE IV

Antigens of Rickettsia prowazeki

	Fraction RF	Fraction RS
	per cent	*per cent*
Nitrogen	10.0	10.0
Reducing sugars (Hagedorn-Jensen)	14.4	15.6
Carbohydrates (orcinol)	8.5	12.5
Amino sugars (as glucosamine)	1.6	1.9

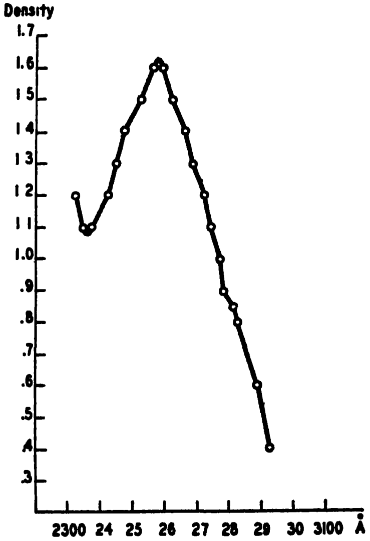

FIG. 3. Ultraviolet absorption spectrum of nucleic acid isolated from rickettsiae by digestion with crystalline trypsin.

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[8] We wish to thank Dr. K. Meyer of this College for the amino sugar determinations.

biuret and Hopkins-Cole tests were positive, the xanthoproteic and Millon tests negative; the ninhydrin reaction was positive in Fraction RF only. Both substances were precipitable with acids and stable to heating and pH changes, similar to the soluble specific material isolated from Fraction A.

Nucleic Acid—When the solution resulting from the treatment of rickettsial organisms with crystalline trypsin was subjected to an electrophoretic separation (Table III, Experiment 3), an intermediate boundary (mobility −14.9 on the descending side) was observed which did not appear in preparations obtained with commercial trypsin. This material could be separated from the fast component, Fraction RF, which it accompanies, by the sedimentation of Fraction RF in the ultracentrifuge. The resulting supernatant exhibited the ultraviolet absorption spectrum characteristic for nucleic acid with a maximum at 2575 A (determined at a concentration of 0.01 mg. of P per cc.). This spectrum is shown in Fig. 3.

DISCUSSION

The first conclusion that may be drawn from the studies here presented is that the antigenic fractions of *Rickettsia prowazeki*, regardless of whether they are liberated from the rickettsiae by tryptic digestion or isolated from certain by-product fluids obtained during the purification of the organisms (Fraction A), occur in a highly polymerized, perhaps particulate, state which permits their separation from accompanying substances in the ultracentrifuge. An inspection of the electrophoresis patterns of different preparations reported in Tables I to III shows them to be composed of varying combinations of five fractions with the following abbreviated mobilities (based on the descending boundaries): Boundary I, −4; Boundary II, −5; Boundary III, −7; Boundary IV, −14; Boundary V, −18. Boundaries I and III probably represent normal yolk sac constituents. The mobility of Boundary II corresponds to that of Fractions AB and AS-S, isolated from the by-product fluid Fraction A, and to Fraction RS, obtained by the tryptic digestion of rickettsial organisms. Boundary IV appears to belong to nucleic acid, or to a fraction consisting predominantly of nucleic acid, which is found in Fraction A (Table II, Experiment 3) and also as a result of the digestion of rickettsiae with crystalline trypsin (Table III, Experiment 3). The very fast component to which Boundary V is assigned (Table III, Fraction RF) has been isolated from rickettsiae following their digestion with proteolytic enzymes.

This is not the place to discuss the immunological properties of rickettsial components; this will doubtless be done at a later date. It may, however, be pointed out that the examination of the rickettsial organisms revealed the presence in them of two main antigenic fractions (Fractions RF and RS), differing in electrophoretic mobility, if not in chemical composition, but similar in the readiness with which they are sedimented in the ultracentrifuge. One of the supernatants obtained in the course of the processing of the rickettsial suspensions, *viz.* Fraction A, has been found to contain a similarly high molecular soluble antigen that in many respects resembled Fraction RS obtained from the rickettsiae themselves. It is not unlikely that this material arises from organisms disintegrating during their cultivation in the growing embryo. The lipids (possibly carried over from the culture medium) and the nucleic acid were devoid of specific serological activity.

Very little can be said at present about the chemical constitution of the antigenic fractions of rickettsiae. They obviously are rich in nitrogenous substances and in carbohydrates and may best be regarded as high molecular carbohydrate-protein complexes, some antigenic groups of which are resistant to tryptic digestion. It should be pointed out that about three-quarters of the total contents of purified rickettsiae was accounted for as two distinct antigenic fractions, in addition to lipids and nucleic acid; but the study of the chemical architecture of these substances will have to await the accumulation of more material.

The authors take pleasure in acknowledging their indebtedness to Colonel H. Plotz and his associates of the Division of Virus and Rickettsial Diseases, Army Medical School, Washington, D. C., without whose help this work could not have been carried out. They are grateful to Dr. D. H. Moore of this College for the ultracentrifuge and electrophoretic separation experiments.

SUMMARY

Formolized preparations of *Rickettsia prowazeki*, obtained from the yolk sac of the developing chick embryo, and the wash fluids of these preparations were subjected to fractionation procedures which permitted the isolation of typhus-specific antigenic substances. Some of the chemical, immunological, and protective properties of these fractions, which were high molecular carbohydrate-protein complexes, are discussed. The isolation of nucleic acid and lipid fractions is likewise recorded.

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THE PARTITION OF URINARY NITROGEN AFTER THE ORAL ADMINISTRATION OF GLUTAMIC ACID, PYRROLIDONECARBOXYLIC ACID, PROLINE, AND HYDROXYPROLINE TO RABBITS

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(Received for publication, May 23, 1944)

Studies of the metabolism *in vivo* of proline and hydroxyproline and of the related pyrrolidonecarboxylic acid are not numerous. Many earlier researches in which attempts were made to demonstrate relationships between these compounds and glutamic acid and ornithine are not convincing because of the fact that the rat growth procedure used at that time has now been shown to be inadequate. More recent experiments on the growth of young white rats have led to the belief that none of these amino acids is an essential component of the diet[1] (1). Proline (3, 4) and hydroxyproline (4) are stated to give rise to essentially the same amounts of extra glucose of the urine when administered to phlorhizinized dogs.

The metabolism of pyrrolidonecarboxylic acid, the lactam of glutamic acid, has been investigated more frequently and with conflicting results (5–10). All are agreed that relatively little extra urea is excreted and that much of the ingested acid may appear unchanged in the urine. Any utilization presumably results from a slow transformation of pyrrolidonecarboxylic acid to glutamic acid, and the deamination of the latter with the formation of urea. However, in much of the work, details are lacking and the methods are not entirely satisfactory. The reverse reaction, the biological conversion of glutamic acid to pyrrolidonecarboxylic acid, has been demonstrated recently (11). When isotopic *d*-glutamic acid was fed to a rat, *d*-pyrrolidonecarboxylic acid was isolated from the urine. Evidence of the biological interconversion of proline, hydroxyproline, ornithine, and glutamic acid has also been obtained in experiments with isotopic proline and a scheme of possible reactions has been presented (12).

In connection with an investigation of urea formation in the intact organism of the rabbit after administration of a considerable number of naturally occurring amino acids, we have made a comparative study of the excretion of urinary nitrogen after proline, hydroxyproline, glutamic acid, and pyrrolidonecarboxylic acid have been fed. We have observed a marked difference in the ability of the animals to metabolize proline and hydroxyproline. The nitrogen of the former amino acid is excreted chiefly as extra urea nitrogen, which is in contrast to the small amounts of extra urea nitrogen excreted after the administration of hydroxyproline. This difference in the ease with which the two prolines are utilized has some parallels in *in vitro* experiments with tissue slices, as will be discussed.

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[1] In a more recent discussion (2), neither the prolines nor glutamic acid is included among the seventeen amino acids which "have been definitely classified with respect to their growth significance," *i.e.* as essential or as dispensable components of the food of the young white rat, although in earlier compilations these three amino acids have been listed as dispensable (1).

EXPERIMENTAL

Source and Purity of Compounds Fed—*l*-Proline and *l*-hydroxyproline, products of the hydrolysis of gelatin, were received from the Department of Chemistry of the University of Illinois. The *l*-glutamic acid, prepared in this laboratory by the hydrolysis of wheat flour, gave no test for either chloride or ammonia. Pyrrolidonecarboxylic acid was made from glutamic acid essentially by the procedure described by Abderhalden and Kautzsch (13). Analyses for nitrogen (macro-Kjeldahl) showed that all the compounds were of satisfactory purity. In addition, the two preparations of the pyrrolidonecarboxylic acid used were tested for amino nitrogen by the gasometric method of Van Slyke. No amino nitrogen could be detected in one preparation, while the second contained 0.09 per cent (equivalent to 0.86 per cent of the total nitrogen). This acid was also titrated with standard alkali, three different indicators being used with satisfactory results.

Determination of Urinary Constituents—Total nitrogen was determined by the usual modified Kjeldahl macromethod; urea nitrogen by the urease method of Van Slyke and Cullen, the ammonia being determined by the usual aeration-titrimetric procedure. Urease was prepared by glycerol extraction of jack bean meal, as described by Koch (14). The partition of urinary sulfur was made by the usual gravimetric methods (Denis-Benedict, Folin). Creatinine was estimated by the micromethod of Folin.

A slight modification of the method proposed by Danielson (15) for the determination of amino nitrogen in blood was employed for the urine analysis. Aliquots of urine (usually 10 to 20 ml.) were treated with permutit to remove ammonia and the supernatant fluids were decanted through glass wool into 100 ml. volumetric flasks. The urines were then made definitely alkaline (phenolphthalein) with 0.1 N sodium hydroxide and the contents of the flask were diluted to volume. The solutions were centrifuged to remove phosphates which would otherwise have caused turbidity in the colorimetric determination. Aliquots (usually 10, 12, and 14 ml.) of the centrifugates were pipetted into 25 ml. volumetric flasks and the color with the naphthoquinonesulfonic acid reagent was developed as described by Danielson. Standards of higher concentration than 0.1 mg. of amino nitrogen were not used, since satisfactory proportionality between standards of 0.1, 0.2, and 0.3 mg. was not always observed. Different amounts of centrifugate (frequently six aliquots) were used instead in order to secure readings of the unknowns comparable to that of the standard.

A standard of glutamic acid rather than the mixed glycine-glutamic acid standard recommended by Danielson was employed, since the color developed by this standard corresponded more closely to those developed by the urines. Proline, as pointed out by Folin (16), gives somewhat more color in the reaction than an equimolar solution of glutamic acid. The urinary values reported in the present study in which proline was fed must therefore represent maximal values, since no correction for the greater color intensity of the reaction with proline was made. Folin did not study the behavior of hydroxyproline in this colorimetric procedure. We have observed that, with hydroxyproline, a yellow-brown color distinct from that given by any of the other amino acids tested develops rapidly after the addition of the naphthoquinonesulfonic acid reagent.[2] Such a prompt development of the color was always obtained in the amino determinations with urines from the experimental periods in which hydroxyproline had been fed and is qualitative evidence of the excretion of hydroxyproline, evidence which is confirmed by the quantitative data obtained. Because of this anomalous behavior of hydroxyproline, this amino acid, rather than glutamic acid, was used as a standard in those periods in which hydroxyproline was administered. Pyrrolidonecarboxylic acid gave no color in the reaction with the naphthoquinonesulfonic acid reagent.

Conduct of Animal Experiments—Quantitative 24 hour specimens of urine were collected from adult male rabbits, fed a commercial rabbit chow in amounts sufficient to maintain their body weight. The amino acids, in amounts equivalent to 0.286 gm. of nitrogen per day, *e.g.* 3 gm. of glutamic acid daily, were fed in aqueous solution through a stomach tube over periods of 2 or 3 days, as shown in Table I.

In some cases the total period of observation extended over 5 months or more (*e.g.*, with Rabbit 5, twelve feeding periods were employed) and the level of nitrogenous metabolism tended to change somewhat over this prolonged period. For this reason, the calculations of extra nitrogen for each experimental period are based on the averages of the two control periods of 3 days each immediately preceding and following the experimental period. The data of Table I represent the amounts of "extra" nitrogen excreted after the administration of the various amino acids. In addition the percentage of the nitrogen fed which is present in the various fractions of urinary nitrogen as extra nitrogen is given. Undetermined nitrogen is calculated as the difference between the total nitrogen and the urea nitrogen. When the excretion of amino nitrogen is great enough to be significant (as on certain of the experimental days), this is added to the urea nitrogen before subtraction from the total nitrogen to obtain the undetermined nitrogen.

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[2] After the present study was completed, Frame, Russell, and Wilhelmi (17) reported a similar rapid development of color in the reaction of both the prolines with the naphthoquinonesulfonic acid reagent. Contrary to the observations of Folin and of ourselves, they noted that both proline and hydroxyproline gave less color (45 and 51 per cent respectively) than either glycine, glutamic acid, or the mixed standard containing an equimolar mixture of these. They employed, however, a modified procedure in which color development was obtained by heating the solution in a boiling water bath for 10 minutes.

TABLE I

Recovery of Extra Urinary Nitrogen after Oral Administration of Glutamic Acid (GA), Pyrrolidonecarboxylic Acid (PCA), Proline (P), and Hydroxyproline (HP)

The compounds were fed over 2 or 3 day periods in amounts equivalent to 0.286 gm. of N per day. Percentage of recovery is calculated as the percentage of N administered recovered as extra N in the various fractions.

Rabbit No.	Period	N fed as		Extra N of urine							
				Total		Urea		Amino acid		Undetermined	
	days		*gm*	*gm.*	*per cent*	*gm.*	*per cent*	*gm*	*per cent*	*gm*	*per cent*
5	3	GA	0.86	0.45	52	0.56	65	0	0	−0.14	
	3	"	0.86	0.79	92	0.78	91	0	0	0	0
	2	"	0.57	0.36	63	0.34	60	0	0	0	0
	3	PCA	0.86	0.70	81	0.46	54	0	0	0.26	30
	2	"	0.57	0.37	65	0.36	63	0	0	0	0
	2	"	0.57	0.27	47	0.12	21	0	0	0.15	26
	2	P	0.57	0.39	68	0.35	61	0.058	10	0	0
	2	HP	0.57	0.55	96	0.35	60	0.193	34	0	0
	2	"	0.57	0.27	47	−0.07		0.219	38	0.09	16
6	3	GA	0.86	0.88	102	1.04	118	0	0	0	0
	3	PCA	0.86	0.46	53	0.36	42	0	0	0.10	12
	3	"	0.86	0.43	50	0.33	38	0	0	0.13	15
	2	"	0.57	0.38	66	0.30	53	0	0	0.12	21
	2	P	0.57	0.46	81	0.50	88	0.033	6	0	0
	2	HP	0.57	0.53	93	0.01	0	0.121	21	0.39	68
	2	"	0.57	0.12	21	−0.16		0.215	38	0	0

Four feeding experiments were carried out with glutamic acid, seven with pyrrolidonecarboxylic acid, three with proline, and five with hydroxyproline. The data from experiments with two of the four animals only are presented in Table I.

l-Glutamic acid appeared to be rapidly deaminized and in none of the experiments was there any significant increase in either urinary amino acid or undetermined nitrogen. Striking increases in urea nitrogen indicated a ready deamination of the glutamic acid and an excretion of the nitrogen as urea nitrogen. In contrast to the metabolic behavior of glutamic acid was that of its lactam, pyrrolidonecarboxylic acid. In the experiments in which this substance was fed, a considerable proportion of the nitrogen administered appeared in the urine as extra undetermined nitrogen and much smaller amounts as urea. In no case was there observed an increase in the amino acid nitrogen of the urine. The marked increase in undetermined nitrogen was observed in six of the seven experiments. Contrary to the observations of previous investigators, no toxicity was observed in any of the experiments with pyrrolidonecarboxylic acid.

It will be recalled that pyrrolidonecarboxylic acid does not react with the naphthoquinonesulfonic acid reagent of Folin, but that after acid hydrolysis, glutamic acid is formed which does react with the reagent. Control experiments with urines to which pyrrolidonecarboxylic and glutamic acids were added showed that the greater part of the nitrogen of pyrrolidonecarboxylic acid could be recovered as amino acid nitrogen after hydrolysis, while there was essentially no change in amino acid nitrogen after hydrolysis of urines to which glutamic acid had been added. Aliquots of the experimental urines after pyrrolidonecarboxylic acid feeding were boiled for 2 hours with concentrated hydrochloric acid, partially decolorized with Lloyd's reagent, and the ammonia removed by permutit. When the amino acid determination was carried out on aliquots thus treated, there was observed a very considerable increase in the amino acid nitrogen as compared with that of the unhydrolyzed urine. This is evidence of the excretion of unchanged pyrrolidonecarboxylic acid in the urine. Since the amount of acid fed was small, no isolation studies were attempted.

Stekol and Schmidt (7) observed a slight decrease in urinary inorganic sulfates after the administration of pyrrolidonecarboxylic acid to dogs and suggested that the acid was conjugated with sulfuric acid and excreted in conjugated form in the bile. In a later study (8), analyses of the bile of rats fed pyrrolidonecarboxylic acid afforded no evidence in support of this hypothesis. We have carried out two experiments with rabbits in which pyrrolidonecarboxylic acid (as the sodium salt) was injected subcutaneously in amounts equivalent to 0.259 and 0.283 gm. of nitrogen respectively. There was no evidence of a change in the distribution of sulfur of the urine. For example, in one experiment, the inorganic sulfate sulfur excretions were 174, 164, and 160 mg. on the fore and after days and 167 mg. on the experimental day. The corresponding values for ethereal sulfate sulfur were 14, 15, and 15 mg., and 13 mg., respectively. These two experiments do not indicate that conjugation with sulfuric acid is a factor in the utilization of pyrrolidonecarboxylic acid by the rabbit.

Marked differences were observed in the partition of urinary nitrogen after feeding proline and hydroxyproline. After administration of proline, a considerable proportion of the nitrogen was excreted as extra urea nitrogen and the increases in amino acid nitrogen were small (10 and 6 per cent of the nitrogen fed, in Table I). After hydroxyproline, on the other hand, little extra urea nitrogen appeared in the urine (none in three of the four experiments in Table I) and the increased amino acid nitrogen was equivalent to from 21 to 38 per cent of the nitrogen fed. Moreover, the rapid and characteristic development of the color with the naphthoquinonesulfonic acid reagent suggested strongly the presence of unchanged hydroxyproline in the urine. We have, in a series of comparable unpublished experiments in this laboratory, studied the partition of the extra urinary nitrogen after the oral administration of a considerable number of amino acids and have never observed in any other series so great an excretion of extra amino acid nitrogen or so striking an absence of increased urea excretion in the urine.

We have also carried out experiments similar to those described but differing in that the amino acids were injected subcutaneously on single days rather than fed over 2 or 3 day periods. The results have been somewhat difficult to interpret, since the total and urea nitrogens were not as uniform as was desired and in some cases the total nitrogen excreted exceeded the amount injected. The changes in amino acid and undetermined nitrogen seem significant. As far as these two fractions of the urinary nitrogen are concerned, the changes observed after the injection of proline and hydroxyproline were comparable to those obtained after oral administration of these amino acids.

A difference in the utilization of proline and hydroxyproline *in vitro* has been demonstrated by Bernheim and Bernheim (18), who observed in experiments with minced rat liver that the rate of oxygen uptake was much slower with hydroxyproline than with proline. In similar experiments with "resting" *Bacillus proteus*, proline was oxidized readily, while with hydroxyproline the oxidation was so slow that no definite oxygen uptake could be demonstrated (19). In experiments with kidney slices, Weil-Malherbe and Krebs (20) found that although both proline and hydroxyproline, in the presence of ammonium salts and oxygen, gave rise to an acid amide, presumably glutamine,[3] proline was oxidized under aerobic conditions more rapidly than was hydroxyproline. Moreover, when the kidney slices were treated with arsenious oxide, the dinitrophenylhydrazone of α-ketoglutaric acid could be isolated as a product of the metabolism of proline. No similar hydrazone could be obtained with hydroxyproline. Neber (21) has isolated glutamic acid from systems containing proline and kidney slices. A notable increase in amino acid nitrogen was observed with proline and significantly less amino acid nitrogen in the one experiment with hydroxyproline recorded.

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[3] Glutamine reacts with the β-naphthoquinonesulfonic acid reagent and gives nearly theoretical values when compared with a glutamic acid standard.

In studies of ketogenesis in which rat liver slices were used, hydroxyproline has been reported as "considerably ketogenic," while the formation of ketone bodies in the presence of proline was no greater than in control experiments in which no amino acids were added (22).

The results of the *in vitro* experiments indicate that the pathways of metabolism of proline and hydroxyproline are not as yet clearly established and are not identical. Pyrrolidonecarboxylic acid (20, 21) and δ-hydroxy-α-aminovaleric acid (21) are probably not intermediates in the conversion of proline to glutamic acid. Proline may give rise to hydroxyproline *in vivo* (12), but the reverse of this reaction is yet to be demonstrated. The studies here reported on the distribution of urinary nitrogen after the administration of the prolines indicate a much less ready urea formation from hydroxyproline than from proline and a much greater excretion of urinary amino nitrogen, presumably the unchanged amino acid, although other amino nitrogen compounds are not ruled out. It is our hope that it may be possible at a subsequent date to continue the study of this problem *in vivo.*

SUMMARY

The naturally occurring stereoisomers of glutamic acid, proline, and hydroxyproline, and inactive pyrrolidonecarboxylic acid (pyroglutamic acid) have been fed to rabbits and the distribution of the "extra" nitrogen excreted has been studied.

After glutamic acid feeding, the extra nitrogen appeared chiefly in the urea fraction, an indication of a ready utilization (deamination) of the acid. The excretion of urea nitrogen after pyrrolidonecarboxylic acid was irregular and not extensive. Much of the "extra" nitrogen appeared in the "undetermined" nitrogen fraction. When proline was fed, urea nitrogen was considerably increased and slight increases only in the amino acid nitrogen fraction of the urine were observed. Urea formation, as evidenced by excretion of urinary urea, occurred less readily after the oral administration of hydroxyproline. A considerable portion (21 to 38 per cent) of the nitrogen fed as hydroxyproline appeared in the urine as extra amino acid nitrogen.

The relation of these observations to studies of the metabolism of the prolines by tissue slices is discussed.

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LETTERS TO THE EDITORS

A NEW CHICK ANTIANEMIC FACTOR*

Sirs:

In the course of studies to improve the microbiological method of Hutchings, Bohonos, and Peterson[1] for the determination of the norit eluate factor (folic acid), we found that liver Fraction L of The Wilson Laboratories contained a growth essential for *Lactobacillus casei* ε in addition to folic acid. This growth essential was adsorbed upon activated charcoal and eluted with ammoniacal ethanol. It was extracted from the acidified eluate with ether. Treatment of the ether extract with H_2O_2 to destroy folic acid greatly increased the potency of the extract.

Later the treatment of pyridoxine with H_2O_2 was found to give a preparation which promoted the same growth response of *Lactobacillus casei* as did the H_2O_2-treated ether extract. A consideration of the products which might be obtained by treating pyridoxine with H_2O_2 led us to believe that the active agent obtained under the experimental conditions was perhaps the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine. This compound has been synthesized by Harris, Stiller, and Folkers.[2] A quantity of the lactone, prepared according to their procedure, promoted the same growth response in *Lactobacillus casei* as did the H_2O_2-treated pyridoxine. It failed to stimulate the tyrosine decarboxylation by *Streptococcus faecalis* obtained by Gunsalus and Bellamy[3] with H_2O_2-treated pyridoxine.

Because the properties of the adsorbable, ether-extractable factor obtained from liver Fraction L were somewhat similar to factor R of Schumacher, Heuser, and Norris[4] and Hill, Norris, and Heuser,[5] studies on chicks

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* This work was supported in part by the establishment of a fellowship at Cornell University by the Western Condensing Company, San Francisco, California.

[1] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).

[2] Harris, S. A., Stiller, E. T., and Folkers, K., *J. Am. Chem. Soc.*, **61**, 1242 (1939).

[3] Gunsalus, I. C., and Bellamy, W. D., *J. Bact.*, **47**, 413 (1944).

[4] Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, **135**, 313 (1940).

[5] Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutr.*, in press.

were undertaken, in which the purified diet of the latter workers was used. Factor S of Schumacher and his associates,[4] freed of factor R and an antianemic factor, was included in the diet. The results of the chick studies are presented in the table.

The results showed that H_2O_2-treated pyridoxine and the synthetic lactone promoted growth in chicks for the 1st week. These growth increases were highly significant. After the 1st week the growth differences tended to disappear because of other deficiencies.

Experiment No.	Treatment	No. of chicks	Weight at 7 days	Significance of difference, t*	Hemoglobin per 100 cc.†	Significance of difference, t*
			gm.		gm.	
1	None	39	56.2		5.4	
	H_2O_2-treated pyridoxine	39	61.3	4.3	8.7	6.4
2	None	29	54.0		4.1	
	H_2O_2-treated pyridoxine	29	61.8	3.4	8.4	6.6
	Synthetic lactone	29	65.1	5.6	7.9	15.3

* When t = 3.4, the odds are 2964:1; when t = 4.3, the odds are greater than 99,999:1.

† Determinations made at 21 days; twenty-nine per lot in Experiment 1 and fifteen, seven, and seventeen respectively in Experiment 2.

The H_2O_2-treated pyridoxine and the lactone also significantly increased the hemoglobin content of the blood. The antianemic properties of the lactone differentiated it from factor R, since this factor has been shown by Hill and his associates[5] not to increase the hemoglobin level. The lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine, therefore, promotes growth and prevents anemia in chicks.

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Received for publication, June 19, 1944

UTILIZATION OF AMINO ACID ENANTIOMORPHS BY LACTOBACILLUS ARABINOSUS AND LACTOBACILLUS CASEI

Sirs:

The synthetic amino acids are available commercially only in the racemic form. Their use as standards in quantitative microbiological determinations (*Lactobacillus arabinosus*, *Lactobacillus casei*) of amino acids in natural products makes it necessary to know whether the unnatural isomer is active for the assay organisms. It has already been established that only the naturally occurring enantiomorph of leucine,[1] isoleucine,[1] valine,[1,2] tryptophane,[3] glutamic acid,[4] and lysine[4] is utilized by *L. arabinosus*. Likewise for *L. casei*, only the *l* or natural form of valine is active.[5] Results with additional amino acids, some of whose optical isomers are not generally available,[6] are given in the accompanying table.

The amino acid assay procedures which have been described were followed.[7] The *l* isomer of each amino acid was used to construct a standard curve from which the relative activities of the *d*, *dl*, or allo forms were read. In the case of phenylalanine, the racemate was used as the standard, since the *l* isomer was not available. It is evident from the table that only the *l* enantiomorph of threonine and methionine is utilized by *L. arabinosus* and the same is true for *L. casei* with phenylalanine, leucine, tryptophane, arginine, and tyrosine, since, within experimental error, either the *dl* form was 50 per cent as potent as the *l* isomer or the available *d* antipode had less than 0.3 per cent the activity of the latter. Therefore, only the naturally occurring enantiomorph of the amino acids so far examined by the various investigators can be metabolized by the *Lactobacilli*.[8] It is noteworthy that *L. arabinosus* resembles the rat[9] in its inability to grow with the *d* or allo forms of threonine.

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[6] We are greatly indebted to Dr. W. C. Rose and Dr. Madelyn Womack of the University of Illinois for the *dl*-arginine, *d*(+)-phenylalanine, *d*(+)- and *l*(−)-methionine, and *d*(+)-tyrosine, and to Dr. E. E. Howe of the Research Laboratory for obtaining these compounds for us.

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[8] Hegsted and Wardwell (*J. Biol. Chem.*, **153**, 167 (1944)) have recently reported that an apparently pure sample of *d*(+)-leucine was slightly active for *L. arabinosus*.

[9] West, H. D., and Carter, H. E., *J. Biol. Chem.*, **122**, 611 (1938).

Amino acid	*L. arabinosus* 17-5			*L. casei*	
	Per cent activity compared to *l* isomer				
	d	*dl*	Allo	*d*	*dl*
Threonine	<0.25	49	<1.0		
Methionine	<0.30	56			
Phenylalanine				0.25	50*
Leucine					48
Tryptophane					50
Arginine					45
Tyrosine				<0.05	

* Calculated on the basis that the *d* isomer is inactive.

Although the *Lactobacilli* are less versatile than animals with respect to the utilization of *d*-amino acids,[10] other microorganisms can synthesize *d*-amino acids,[11] oxidatively deaminate them,[12] or use them as a source of nitrogen for growth.[13]

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Received for publication, July 11, 1944

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THE ANTAGONISTIC EFFECT OF PHOSPHOLIPIDS ON THE ANTIBACTERIAL ACTION OF PROPAMIDINE

Sirs:

Snell[1] in a recent communication has shown that bacteriostasis produced by propamidine (4,4′-diamidinodiphenoxypropane), a drug shown to be effective in the local chemotherapy of wounds and burns,[2] can be lessened or completely reversed by the addition of certain polyamines. In work carried out in this laboratory it has been found that both the inhibitory and "killing" action of propamidine can be reversed by certain phospholipids, which have been shown by Baker, Harrison, and Miller[3] to prevent the inhibition of bacterial metabolism of synthetic anionic and cationic detergents.

TABLE I

Antagonistic Effect of Soya Lecithin on "Killing" Action of Propamidine on Staphylococcus aureus

Time of exposure, hrs.	Concentration of lecithin, mg. per cc.			
	75	50	25	0
1	+	+	0	0
2	+	0	0	0
3	+	0	0	0
4	+	0	0	0
5	+	0	0	0
7	+	0	0	0

0, no growth in subculture; +, growth in subculture.

In studies in connection with the prevention of the killing effect, 1.5 cc. of the lecithin suspension in phosphate buffer at pH 7.4 and 0.5 cc. of a propamidine[4] solution (10 mg.) were inoculated with 0.2 cc. of a 20 hour broth culture of *Staphylococcus aureus* and subcultures made periodically, as indicated in Table I. Subculturing technique was used to eliminate bacteriostasis. Throughout the experiment the medication tubes were maintained at 37.0°±0.1°.

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[3] Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, **74**, 621 (1941).

[4] The propamidine used in these experiments was in the form of the dihydrochloride salt and was synthesized in these laboratories by the general method of Ashley *et al.* (*J. Chem. Soc.*, 103 (1942)).

In demonstrating reversal of inhibition (Table II), 1.0 cc. of the lecithin suspension and 1.0 cc. of propamidine solution of the concentration necessary to furnish the final concentration indicated were added to sterile plates along with 18 cc. of agar. After solidification the plates were streaked with two loops of a 20 hour broth culture of the organism. Plates were read after 48 hours incubation. Similar antagonisms have been demonstrated with egg lecithin (Merck). The antibacterial actions against the Gram-negative organism, *Escherichia coli*, may similarly be reversed.

TABLE II

Antagonistic Effect of Soya Lecithin on Inhibitory Action of Propamidine on Staphylococcus aureus

Soya lecithin, mg.	(Mg. propamidine)/(20 cc. agar)		
	0.5	1.0	2.0
0	0	0	0
10	+	0	0
20	++	0	0
40	+++	++	0
60	+++	+++	0
80	+++	+++	++
100	+++	+++	+++

0, complete inhibition; +, nearly complete inhibition; ++, slight inhibition; +++, no inhibition; growth = control.

The results of these experiments demonstrate that the phospholipids used are completely capable of protecting the organisms studied against the inhibitory and the killing action of propamidine. The quantitative nature of the relation between the phospholipid and propamidine concentration is demonstrated in Table II.

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Received for publication, July 14, 1944

THE RÔLE OF PANTOTHENIC ACID IN THE SYNTHESIS OF TRYPTOPHANE*

Sirs:

Pantothenic acid has been known to function as a growth vitamin for several bacteria.[1] The biochemical nature of this function is not known. In the course of a study on the growth requirements of various toxigenic strains of *Staphylococcus aureus*, it was observed that certain strains (B-523S and B-523C) required the addition of *tryptophane* to a tryptophane-free basal medium, consisting of vitamin-free casein hydrolysate or of a mixture of amino acids with or without glucose. Riboflavin, nicotinamide, thiamine, *Lactobacillus casei* factor, biotin, and pyridoxine were found to be inadequate to support growth in the absence of tryptophane with or without glucose. On the other hand pantothenic acid supported growth (see the table) in the tryptophane-free medium *in the presence of glucose but not*

Medium	Turbidity readings	
	Series A*	Series B*
Casein hydrolysate	0	27
+ pantothenate 1 γ per ml.	0	27
+ glucose, 0.5%	0	93
+ glucose + pantothenate (as above)	27	90
Synthetic medium	0	52
+ pantothenate 1 γ per ml.	0	54
+ glucose, 0.5%	0	60
+ glucose + pantothenate	49	58

Casein Hydrolysate—M/30 phosphate solution (pH 7.4) of 1 per cent casein hydrolysate (vitamin-free, Smaco) + 9.0×10^{-4} M cysteine hydrochloride + 2.5×10^{-4} M ferrous ammonium sulfate + 3.3×10^{-4} M magnesium sulfate + 1 γ per ml. of medium each of *thiamine chloride* and *nicotonamide*.

Synthetic Medium—In the synthetic medium were included all the constituents as above, except that 1 per cent casein hydrolysate was substituted by a mixture of amino acids: alanine, valine, leucine, glycine, proline, oxyproline, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, hydrochlorides of histidine and lysine, threonine, and isoleucine.

* Series A contains no added tryptophane; Series B contains 1×10^{-4} M added tryptophane.

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* The present study was conducted under grants from the Josiah Macy, Jr., Foundation.

[1] Williams, R. J., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, 3, 253 (1943).

in its absence (the observation period of growth from 2 to 7 days). It is concluded that pantothenic acid mediates the metabolism of glucose leading to or involved in the synthesis of tryptophane and thereby of the growth of staphylococci. These staphylococcal strains appear therefore to be incapable of the synthesis of pantothenic acid and thereby of tryptophane.

It has been previously demonstrated that staphylococci oxidize tryptophane to arylamines of unknown constitution.[2] In this connection it was observed that upon the addition of pantothenic acid arylamines were formed in a basal medium containing glucose. The controls under similar conditions showed neither growth nor arylamine formation. It is also to be noted that sulfonamides influence the metabolism of tryptophane by *Staphylococcus aureus*.[3] Pantothenic acid alone, or in combination with riboflavin, completely or partially abolished the inhibitory action of sulfonamides.[3] This finding is interpreted as additional evidence for the synthesis of tryptophane under the influence of pantothenic acid.

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Received for publication, June 19, 1944

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THE RÔLE OF CRYSTALLINE VITAMIN B_6 IN THE NUTRITION OF THE CHICK

Sirs:

We have reported[1] that, when the crystalline B_6 vitamin is incorporated in the basal chick ration, it has a positive influence on growth (body weight and feathering) and on the blood cell components.

In order to circumvent, in part at least, the possible influence that vitamin B_6 may have on the synthesis of other factors by the intestinal bacterial flora (which Briggs *et al.*[2] refer to as a source of additive factors—B_{10} and B_{11}) some of the same lot of the pure vitamin B_6 crystals was given for 4 weeks, both orally and subcutaneously, as supplement to the basal ration. The data for the two groups and the controls are given in the accompanying table.

Group	Vitamin B_6 per chick per day	No. of chicks		Body weight	Red blood cells	Hematocrit	Hb	Leucocytes	Thrombocytes
		Started	Surviving						
	γ			*gm.*	*millions per c.mm.*	*vol. per cent*	*gm. per cent*	*per c.mm.*	*per c.mm.*
Basal B_6-free ration	None	36	15	89	0.70	11.3	3.38*	8,460	14,690
Normal broiler ration		20	20	207	2.27	31.2	7.74	29,935	31,180
Crystalline B_6, No. 37933 (from yeast) given by pipette	1	13	9	101	1.46	22.1	5.30	17,900	30,300
	4	13	8	157	1.63	24.4	6.33	15,900	29,380
	20	9	7	191	2.25	33.0	8.77	19,910	34,430
Crystalline B_6, No. 37933 (from yeast) injected subcutaneously	1	11	9	122	1.59	24.7	6.44	8,420	23,620
	4	11	9	173	2.00	28.6	7.95	23,210	36,100
	20	6	6	214	2.40	33.8	8.49	25,730	35,180

* Five chicks for this group.

The evidence shows that when the vitamin is given parenterally it has the same biological effect on the chick as it does when given orally (by pipette or in the ration[1]). This finding tends to suggest that the intestinal flora is very probably not influenced, directly at least, by the vitamin and that the effect on growth (both weight and feathering) and blood cell components is due to the vitamin B_6 *per se*. This does not preclude the fact, however, that the digestive juices may serve as intermediary agents and carry some factor into the intestinal tract. If other unknown dietary factor or factors are necessary for growth then, in order to demonstrate their existence, suboptimum rather than optimum levels of vitamin B. must be present in the ration.

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Received for publication June 19 1944

REVERSIBILITY OF THE PHOSPHOROCLASTIC SPLIT OF PYRUVATE

Sirs:

The "hydroclastic" split whereby pyruvic acid is split into formic and acetic acids is a common reaction in certain bacteria. Recently it has been shown by use of *Escherichia coli*[1] that this reaction is really a phosphoroclastic split with acetyl phosphate and formic acid arising from pyruvic acid:

$$\underset{\text{Pyruvic acid}}{CH_3COCOOH} + H_3PO_4 \rightleftharpoons \underset{\text{acetyl phosphate}}{CH_3COO(PO_3H_2)} + \underset{\text{formic acid}}{HCOOH}$$

Acetic acid is formed by the dephosphorylation of acetyl phosphate.

With the aid of C^{13} as a tracer, it has now been possible to show the reversibility of the phosphoroclastic reaction. An enzyme preparation obtained by grinding *Escherichia coli* with powdered glass was incubated with formic acid containing an excess of C^{13} and with pyruvic acid containing normal amounts of C^{13}. At the end of 60 minutes, the unchanged pyruvic acid contained a considerable excess of C^{13} in the carboxyl group. The C^{13} in the carboxyl group and in the residual formic acid were approximately equal, showing that an equilibrium has been reached. Since the formic acid was not in equilibrium with CO_2 as evidenced by the very small excess of C^{13} in the CO_2, C^{13} did not enter into the pyruvic acid by means of a combination of CO_2 and some other compound.

Reversibility of Phosphoroclastic Reaction in Escherichia coli

Products	Excess C^{13}, atoms per cent
CO_2	0.06
—COOH of pyruvic acid	0.80
HCOOH (contained 1.79% excess C^{13})	0.79

The pyruvic acid was degraded with ceric sulfate in the cold after the volatile acids had been removed by steam distillation.

Reversibility of the phosphoroclastic reaction provides another pathway whereby $C^{13}O_2$ can become integrated into organic molecules. Previously such fixation has been assumed to take place only by means of union of a 3-carbon compound and CO_2.[2] CO_2 is normally in equilibrium with formic acid in *Escherichia coli*, although this is not the case in this enzyme preparation. If formic acid is in equilibrium with both pyruvic acid and CO_2, $C^{13}O_2$ can be fixed in the carboxyl group of pyruvic acid and the latter compound can undergo further transformations. With whole cells of *Escherichia coli* such a reaction actually occurs. Although the phosphoroclastic reaction is limited to a relatively small number of bacterial species, the reaction is similar to other reactions in which acetic acid and CO_2 are formed from pyruvic acid and it is possible that these reactions may prove reversible also.

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Received for publication, July 18, 1944

KETO ACID FORMATION THROUGH THE REVERSAL OF THE PHOSPHOROCLASTIC REACTION*

Sirs:

The oxidation-reduction potential of the system, (pyruvate-water)/(acetate-CO_2-H_2), is about 0.3 volt negative to the hydrogen potential ($-\Delta F_0$, 15 kilocalories). Consequently the release of hydrogen and carbon dioxide from pyruvate hydrate occurs with great energy loss and is essentially irreversible. The finding, however, of acetyl phosphate as the dehydrogenation product of pyruvate[1] changed the aspect of irreversibility of dehydrogenative decarboxylation. Through the substitution of phosphate for water a migration of more than 12 kilocalories into the carboxyl phosphate bond is effected. This reduces the external energy loss from 15 to less than 3 kilocalories. Therefore, the phosphoroclastic system, (pyruvate-phosphate)/(acetyl phosphate-CO_2-H_2), becomes essentially reversible.

The finding of Utter and Werkman[2] that in extracts of *Escherichia coli* acetyl phosphate and formate are formed from pyruvate offered a very simple reaction system to test for reversibility. The energy equivalence of formic acid with a mixture of molecular hydrogen and carbon dioxide made the results with this system automatically transferable to the more complicated hydrogenative carboxylation of acetyl phosphate with molecular hydrogen or suitable hydrogen donor.

The bacteriological material was kindly supplied by Professor Werkman. Extracts of *Escherichia coli* were incubated anaerobically with acetyl phosphate and formate. Sodium fluoride (0.03 M) was added to delay rapid enzymatic split of acetyl phosphate. To determine the small amounts of keto acid which appeared on incubation the sensitive colorimetric method of Friedemann and Haugen[3] was used in the modification specific for pyruvate.

A typical experiment is shown in the accompanying table. Apparently a gradual decomposition of acetyl phosphate, after an initial rise, causes the keto acid level later to fall parallel with acetyl phosphate disappearance. On the other hand, acetyl phosphate without formate did not give rise to keto acid. When pyruvate was added at the start in slight excess of the equilibrium concentration, the keto acid concentration leveled off

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* This work was aided by a grant of the Commonwealth Fund.

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Incubation time	Formate	Acetyl phosphate	Pyruvate
min.	*micromoles per ml.*	*micromoles per ml.*	*micromole per ml.*
0	150	50	0.03
25	150	38	0.17
60	150	24	0.14
60		24	0.03
150	150	2	0.07

at equilibrium level only with formate *and* acetyl phosphate present. These experiments allow an approximation of the equilibrium constant, amounting to about 10^{-2} for the reverse phosphoroclastic reaction.

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Received for publication, July 18, 1944

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